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(54) Title: TISSUE-DERIVED TUMOR GROWTH INHIBITORS, METHODS OF PREPARATION AND USES THEREOF

(57) Abstract

The present invention provides (1) an antibody which (a) specifically binds to human TGF-β3 and (b) exhibits substantially no cross reactivity with TGF-β1 or TGF-β2 and (2) antibodies directed against the pro region of the TGF-β precursor. Further, this invention provides a pharmaceutical composition comprising the pro region of the TGF-β precursor. Also, this invention provides methods for diagnosing, detecting and treating subjects suffering form disorders associated with TGF-β3.

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TISSUE-DERIVED TUMOR GROWTH INHIBITORS, METHODS OF PREPARATION AND USES THEREOF

This application is a continuation-in-part of U.S. Serial No. 353,410, filed May 17, 1989, which is a continuation-in-part of U.S. Serial No. 183,224, filed April 20, 1988, which is a continuation in part of U.S. Serial No. 111,022, filed October 20, 1987, which is a continuation-in-part of U.S. Serial No. 922,121, filed October 20, 1986, now abandoned, which was a continuation-in-part of U.S. Serial No. 847,931, filed April 7, 1986, now abandoned, which was a continuation-in-part of U.S. Serial No. 725,003, filed April 19, 1985, now abandoned, the contents of each are hereby incorporated by reference into the present application.

Background of the Invention

Throughout this application, various publications are referenced by Arabic numerals within parentheses. Full citations for these publications may be found at the end of the specification immediately preceding the claims. The disclosures of these publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art as known to those skilled therein as of the date of the invention described and claimed herein.

Transforming growth factor β (TGF-β) is part of a family of multifunctional proteins which appear to modulate, alone or in combination with other molecules, cell proliferation and differentiation. Reportedly, TGF-β, which comprises a mature a precursor and a pro region of the precursor form, contains a subclass of molecules designated TGF-β1, -β2, -β3, -β4, and-β5 (24, 25, 26).

Mature TGF-ß has been isolated from various species. Murine, bovine, human, and porcine TGF-ß have been isolated and show very little difference in amino acid composition (26, 27, 28, 29).

The cDNA sequence of mature TGF-ß, its expression in both normal and transformed cells, and methods of producing biologically active mature TGF-ß in eucaryotic cells have been described (26, 28, 29, 54, 55).

Antibodies directed to mature TGF-ß1 and -ß2 have previously been described (32, 33, 34, 35, 36). Because of the high homology between the various isoforms of mature TGF-ß, these antibodies exhibit substantial cross reactivity. Antibodies which are specifically directed to human mature TGF-ß3 and exhibit no substantial cross reactivity with other TGF-ß3 isoforms have not been described.

Summary of the Invention

The present invention provides (1) an antibody which (a) specifically binds to human TGF-β3 and (b) exhibits substantially no cross reactivity with TGF-β1 or TGF-β2 and (2) antibodies directed against the pro region of the TGF-β precursor. Further, this invention provides a pharmaceutical composition comprising the pro region of the TGF-β precursor. Also, this invention provides methods for diagnosing, detecting and treating subjects suffering from disorders associated with a TGF-β.

Brief Description of the Figures

Figure 1 shows the nucleotide sequence encoding TGF-β3 and its deduced amino acid sequence. Putative glycosylation sites and polyadenylation signals are underlined. The start of the mature TGF-β3 is marked by an asterisk at nucleotide positions 1163-1165.

Figure 2 is a schematic representation of the construction of the pCMV-TGF-β3 expression plasmid from pORFX and pBlue-TGF-β3 plasmids.

Figure 3 shows the level of TGF-β3 mRNA expression, determined by Northern hybridization using a TGF-β3 specific probe, of parental CHO cells (lane 1), CHO cells transfected with TGF-β3 cDNA (CHO 6.35) (lane 2) and CHO 6.35 amplified with 20nM MTX (CHO 6.35/20nM (lane 3).

Figure 4 shows a schematic diagram of mRNA encoding TGF-β3 with the coding sequence boxed. The relative extension of the cDNA inserts obtained from placenta (1.7 kb), umbilical cord (1.9 kb) and A673 (1.7 kb) libraries is indicated. The dashed part of the box represents the C-terminal region showing high homology to TGF-βs. The 5' EcoRI-Bg II restriction fragment of the placenta cDNA is indicated by a bar.

Figure 5

(A) shows the dose response of mink cell growth inhibition using purified TGF-β1. Cell growth was quantitated by the metabolism of MTT 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetraazolium bromide; Thiazolyl blue).

(B) shows the dose response of mink cell growth inhibition using acid activation serum free supernatants CHO 6.35/20nM transfectant and CHO 6.35 transfectant. Cell growth was quantitated by the metabolism of MTT.

Figure 6 shows the relative location of the various TGF-β3 peptides used as antigens.

Figure 7 shows the immunoprecipitation of native recombinant TGF-β3 protein by β3V antibody.

Figure 8

(A) shows the immunoblot of TGF-β3 from conditioned media of CHO 6.35/20nM transfectant using β3III and β3V antibodies for detection from gels under reducing conditions.

(B) shows the immunoblot of TGF-β3 from conditioned media of CHO 6.35/20nM transfectant using β3III and β3V antibodies for detection from gels under non-reducing conditions.

Figure 9 shows a Western blot of cell extract (9A) and conditioned media (9B) of the CHO 6.35/20nM transfectant using β3V antibody for detection.

Figure 10 A, B, C, and D shows the staining to paraffin sections of human umbilical cord by β3V antibody and control antibody. A and C show fibroblast and epithelial staining and smooth muscle fiber staining, respectively, by β3V antibody. B and D show no staining by control rabbit polyclonal antibody.

Figure 11 A, B, and C shows specific neutralization of TGF-β3 inhibition of mink cell growth by the antibody ß3V.

Figure 12: Effect of TGF-β3 (closed circles) and TGF-β1 (open circles) on DNA synthesis, collagen synthesis and alkaline phosphatase activity. Osteoblast-enriched cultures from fetal rat parietal bone were cultured to confluence and then serum deprived for 20 hours prior to a 23 hours treatment with either TGF-β3 or TGF-β1 at the concentrations shown.

(A) DNA synthesis rates were measured by labelling cells with

[^{3}H]thymidine for the last 2 hours of culture; acid-insoluble material was assayed by scintillation counting.

(B) Collagen synthesis was measure by labeling with [^{3}H]proline the last 2 hours of culture; acid-insoluble cell extracts were digested with nonspecific protease-free bacterial collagenase and radioactivity was determined in the enzyme-released supernatants.

(C) Alkaline phosphatase activity was measured in cell extracts by hydrolysis of p-nitrophenyl phosphate to p-nitrophenol (PNP). Data are the means ± SEM of four to six replicate cultures per condition.

Figure 13: Inhibition of hematopoietic stem cells by TGF-β3. Primary bone marrow cells were enriched for a progenitor stem cell population by immunodepletion and cultured as described. Cells were grown in the presence of Mo-T Cell conditioned media and increasing concentrations of purified TGF-β3 (0, 10, 100, 1000) and colony sizes determined at 3, 7 and 14 days. TGF-β3 inhibited proliferation and subsequent increase in hematopoietic colony size in a dose and time dependent manner.

Figure 14: Evaluation of TGF-β3 as an chemoprotective agent in vitro - Mink cells were seeded in 96-well plates at 10^3 cells/well in 100 µl of DMEM supplemented with 10% fetal bovine serum. Well's containing treated cells received 25 µl of TGF-β3 (50ng/ml). After 24 hr incubation with TGF-β3, 25 µl of colchicine or vinblastine was added. After another 24 hrs, the media was removed and the cells washed once with Dulbecco's PBS and fresh complete media added. The cells were incubated for another 7 days. Cell growth was quantitated by uptake of ^{125}I-iodo-2'deoxyuridine as previously described.

Detailed Description of the Invention

In accordance with the invention, mature TGF-β3 is defined as a recombinant homodimeric protein which comprises two polypeptides each of which consists essentially of 112 amino acids and has a sequence substantially identical to the amino acid sequence shown in **Figure** 1 beginning with an alanine encoded by nucleotides 1163-1165 and ending with a serine encoded by nucleotides 1496-1498.

Moreover, as used herein TGF-β3 precursor is a recombinant homodimeric protein which comprises two polypeptides, each polypeptide encoded by a sequence substantially identical to the amino acid sequence shown in **Figure** 1 beginning with a methionine encoded by nucleotides 263-265 and ending with a serine encoded by nucleotides 1496-1498.

Further, as used herein the pro region of the TGF-β3 precursor is a recombinant protein which comprises the TGF-β3 precursor without the mature TGF-β3. Additionally, the pro region of the TGF-β3 precursor is a protein region encoded by a sequence substantially identical to the amino acid sequence shown in **Figure** 1 beginning with a methionine encoded by nucleotides 263-265 and ending with an arginine encoded by nucleotides 1160-1162.

Also, as used herein, reference to TGF-β means either mature TGF-β (e.g. TGF-β1, -β2, -β3), TGF-β precursor (e.g. TGF-β1 precursor, TGF-β2 precursor, TGF-β3 precursor), or the pro region of the TGF-β (e.g. TGF-β1, -β2, -β3) precursor.

Antibodies directed to mature TGF-β have previously been described. However, these antibodies exhibit substantial cross reactivity to various TGF-β isoforms because of the high

homology between them. Surprisingly, the antibody, disclosed herein, is specifically directed to mature TGF-ß3 and displays no substantial cross reactivity with other TGF-ß isoforms.

The present invention provides an antibody (for example a monoclonal or a polyclonal antibody) which (a) specifically binds to human TGF-ß3 and (b) exhibits substantially no cross reactivity with TGF-ß1 or TGF-ß2. In one embodiment of the invention, the antibody may be directed to an epitope defined by the amino acid sequence DTNYCFRNLEENC. In another embodiment, the subject antibody is directed to an epitope defined by the amino acid sequence YLRSADTTHSTVLGLYNTLNPEASASY.

Generally, an antibody comprises two molecules, each molecule having two different polypeptides, the shorter of which functions as the light chains of the antibody and the longer of which polypeptides function as the heavy chains of the antibody. However, as used herein, antibody is given a functional definition, i.e. any molecule, whether naturally-occurring, artificially induced, or recombinant, which has specific immunoreactive activity. Normally, as used herein, an antibody will include at least one variable region from a heavy or light chain (37-43).

Accordingly, a fragment of a naturally occurring or recombinant antibody molecule is encompassed within the scope of this invention. As used herein a Fab protein or a F(ab')2 protein which exhibits immunoreactive activity is an antibody.

This invention further provides an antibody which (a) specifically binds to a pro region of the TGF-ß3 precursor and (b) exhibits substantially no cross reactivity with mature TGF-ß3.

In one example of the invention, the subject antibody which (a) specifically binds to a pro region of the TGF-β3 precursor is directed to an epitope defined by the amino acid sequence GDILENIHEVMEIKRKGVDNEDD (Table 1). In another example, the subject antibody is directed to an epitope defined by the amino acid sequence GDILENIHEVMEIK (Table 1). In yet a further example, the subject antibody is directed to an epitope defined by the amino acid sequence EEMHGEREEGCTQENTESEY (Table 1) .

Additionally, the present invention provides a method of detecting a TGF-β3 precursor from a sample. The method comprises contacting the sample with a suitable amount of the above-described antibody, under conditions such that the antibody binds to the TGF-β3 precursor and detecting the antibody bound to the TGF-β3 precursor and thereby detecting the TGF-β3 precursor from the sample.

Further, the present invention provides a method of detecting a pro region of a TGF-β3 precursor from a sample which comprises contacting the sample with a suitable amount of the above-described antibody, under conditions such that the antibody binds to the pro region of the TGF-β3 precursor and detecting the antibody bound to the pro region of the TGF-β3 precursor and thereby detecting the pro region of the TGF-β3 precursor from the sample.

The present invention also provides a method of diagnosing a disorder associated with a variation in a TGF-β levels in a human subject. The method comprises (1) obtaining a sample from the subject, (2) detecting the presence of the TGF-β in the sample, and (3) determining the amount of TGF-β in the sample thereby diagnosing the disorder. In accordance with the invention, the disorder may be any disorder selected from a group including, but not limited to, osteoporosis, an

immunosuppressive disease, a bone disorder, an AIDS viral infection, a dermatological disorder, myocardial ischemia, a myopathic disorder, a connective tissue disorder, or a neurological disorder.

Further, in accordance with the above-described method, the TGF-β may be TGF-β1. Alternatively, the TGF-β may be TGF-β2. Further alternatively, the TGF-β may be TGF-β3. TGF-β3 is preferred.

In one example of the above-described method, when TGF-β is mature TGF-β3, detection of the variation in mature TGF-β3 levels may be effected by an antibody which specifically binds to mature TGF-β3 and exhibits substantially no cross reactivity with mature TGF-β1 or mature TGF-β2. Alternatively, detection may be effected by an antibody which specifically binds to the pro region of human TGF-β3 precursor and exhibits substantially no cross reactivity with mature TGF-β3.

TGF-β3 is a bifunctional growth factor. The experiments disclosed herein illustrate that TGF-β3 inhibits or stimulates the same target cell depending upon the quality of exposure to and concentration of other exogenous factors. As a potent modulator of cell growth and differentiation, the regulation of TGF-β3 levels in concert with other exogenous factors is important for normal tissue function and development.

This invention provides a method for treating a subject suffering from a disorder associated with a TGF-β which comprises administering to the subject an amount of an antibody which specifically recognizes TGF-β and neutralizes TGF-β activity.

In accordance with the above-described invention, the method

includes treating a subject suffering from cancer which comprises administering to the subject an amount of an antibody which specifically recognizes TGF-ß and neutralizes TGF-ß activity.

Additionally, the method includes treating a subject suffering from arthritis which comprises administering to the subject an amount of an antibody which specifically recognizes TGF-ß and neutralizes TGF-ß activity.

Further, this method further includes treating a subject suffering from an immune-suppressive disease which comprises administering to the subject an amount of an antibody which specifically recognizes TGF-ß and neutralizes TGF-ß activity.

The subject method also includes treating a subject suffering from an AIDS viral infection. The method comprises administering to the subject an amount of an antibody which specifically recognizes TGF-ß and neutralizes TGF-ß activity.

Also, the method includes treating a subject suffering from myocardial ischemia. The method comprises administering to the subject an amount of an antibody which specifically recognizes TGF-ß and neutralizes TGF-ß activity.

This method further includes treating a subject suffering from a myopathic disorder which comprises administering to the subject an amount of an antibody which specifically recognizes TGF-ß and neutralizes TGF-ß activity.

Also, this method includes treating a subject suffering from a connective tissue disorder which comprises administering to the subject an amount of an antibody which specifically recognizes TGF-ß and neutralizes TGF-ß activity.

Additionally, this method includes treating a subject suffering from a atherosclerosis. The method comprises administering to the subject an amount of an antibody which specifically recognizes TGF-ß and neutralizes TGF-ß activity.

The present method includes treating a subject suffering from a neurological disorder which comprises administering to the subject an amount of an antibody which specifically recognizes TGF-ß and neutralizes TGF-ß activity.

Additionally, the method includes treating a subject suffering from a bone disorder which comprises administering to the subject an amount of an antibody which specifically recognizes TGF-ß and neutralizes TGF-ß activity.

In one example of the above-described methods of treatment, the antibody (e.g. a polyclonal, preferably a monoclonal antibody) is an antibody which specifically recognizes mature TGF-ß and exhibits substantially no cross reactivity with the pro region of the TGF-ß precursor. In another example, the antibody is an antibody which specifically binds to mature TGF-ß3 and exhibits substantially no cross reactivity with mature TGF-ß1 or mature TGF-ß2. In another example of the invention, the antibody may be a humanized antibody. In alternative examples, the antibody may be in the form of a F(ab) fragment or a F(ab')2 fragment.

As used herein, a humanized antibody includes structurally engineered antibodies comprising a polypeptide containing a human constant region or engineered such that they are made non-immunogenic in humans by one skilled in the art (37, 38, 39, 40, 41, 42, 43).

The present invention also provides a pharmaceutical composition comprising an effective amount of a pro region of

a TGF-ß precursor and a suitable pharmaceutical carrier. In one example of the subject pharmaceutical composition the pro region of the TGF-ß precursor is the pro region of the TGF-ß1 precursor. Alternatively, in another example the pro region of the TGF-ß precursor is the pro region of the TGF-ß2 precursor. In a preferred example, the pro region of the TGF-ß precursor is the pro region of the TGF-ß3 precursor.

Moreover, this invention also provides a method for treating a subject suffering from a cancer which comprises administering to the subject an amount of the above-described pharmaceutical composition so as to alleviate the symptoms of the cancer and thereby treating the subject.

Also, the invention provides a method for treating a subject suffering from a connective tissue disorder which comprises administering to the subject an amount of the above-described pharmaceutical composition so as to alleviate the symptoms of the disorder and thereby treating the subject.

The invention additionally provides a method for treating a subject suffering from a neurological disorder which comprises administering to the subject an amount of the above-described pharmaceutical composition so as to alleviate the symptoms of the disorder and thereby treating the subject.

Also, this invention provides a method for treating a subject suffering from an immunosuppressive disorder which comprises administering to the subject an amount of the previously-described pharmaceutical composition so as to alleviate the symptoms of the disorder and thereby treating the subject.

Furthermore, the invention provides method for treating a subject suffering from a bone disorder associated with a TGF-ß which comprises administering to the subject an amount of the

previously-described pharmaceutical composition so as to alleviate the symptoms of the disorder and thereby treating the subject.

Additionally, the present invention also provides a method for treating a subject suffering from myocardial ischemia which comprises administering to the subject an amount of the previously-described pharmaceutical composition so as to alleviate the symptoms of myocardial ischemia and thereby treating the subject.

This present invention also provides a method for treating a subject suffering from a myopathic disorder which comprises administering to the subject an amount of the above-described pharmaceutical composition so as to alleviate the symptoms of the disorder and thereby treating the subject.

Additionally, the invention provides a method for treating a subject suffering from atherosclerosis. The method comprises administering to the subject an amount of the above-described pharmaceutical composition so as to alleviate the symptoms of atherosclerosis and thereby treating the subject.

Also, this invention provides a method for treating a subject suffering from arthritis. The method comprises administering to the subject an amount of the above-described pharmaceutical composition so as to alleviate the symptoms of arthritis and thereby treating the subject.

This invention further provides a method for treating a subject suffering from an AIDS viral infection. The method comprises administering to the subject an amount of the above-described pharmaceutical composition so as to alleviate the symptoms of the infection and thereby treating the subject.

Further, this invention additionally provides a method for treating a subject suffering from a disorder associated with a TGF-ß which comprises administering to the subject an amount of mature TGF-ß3 so as to alleviate the symptoms of the disorder and thereby treating the subject.

In accordance with the practice of the invention, the method includes treating a subject suffering from a connective tissue disorder. The method comprises administering to the subject an amount of mature TGF-ß3 so as to alleviate the symptoms of the disorder and thereby treating the subject.

Moreover, the method also includes treating a subject suffering from a neurological disorder which comprises administering to the subject an amount of mature TGF-ß3 so as to alleviate the symptoms of the disorder and thereby treating the subject. In one example, the neurological disorder may be a demyelinating disease.

This method further includes treating a subject suffering from an immunosuppressive disorder which comprises administering to the subject an amount of mature TGF-ß3 so as to alleviate the symptoms of the disorder and thereby treating the subject.

The present method also includes treating a subject suffering from an inflammatory disorder. The method comprises administering to the subject an amount of mature TGF-ß3 so as to alleviate the symptoms of the disorder and thereby treating the subject.

This method includes treating a subject suffering from septic shock. The method comprises administering to the subject an amount of mature TGF-ß3 so as to alleviate the symptoms of septic shock and thereby treating the subject.

This method also includes treating a subject suffering from a bone disorder which comprises administering to the subject an amount of mature TGF-β3 so as to alleviate the symptoms of the disorder and thereby treating the subject. Further, in accordance with the claimed method, the bone disorder may be a bone fracture.

Furthermore, the method includes treating a subject suffering from a dermatological disorder which comprises administering to the subject an amount of mature TGF-β3 so as to alleviate the symptoms of the disorder and thereby treating the subject.

The method additionally includes treating a subject suffering from myocardial ischemia which comprises administering to the subject an amount of mature TGF-β3 so as to alleviate the symptoms of myocardial ischemia and thereby treating the subject.

Additionally, the method also includes treating a subject suffering from a myopathic disorder which comprises administering to the subject an amount of mature TGF-β3 so as to alleviate the symptoms of the disorder and thereby treating the subject.

The present method additionally includes treating a subject suffering from atherosclerosis. The method comprises administering to the subject an amount of mature TGF-β3 so as to alleviate the symptoms of atherosclerosis and thereby treating the subject.

Also, the method includes treating a subject suffering from an AIDS viral infection. The method comprises administering to the subject an amount of mature TGF-β3 so as to alleviate the symptoms of the AIDS viral infection and thereby treating the subject.

Also, the present invention concerns a method for treating a subject suffering from a disorder associated with a TGF-ß which comprises administering to the subject an amount of TGF-ß3 precursor so as to alleviate the symptoms of the disorder and thereby treating the subject.

In accordance with the practice of the subject invention, the method includes treating a subject suffering from a cancer which comprises administering to the subject an amount of TGF-ß3 precursor so as to alleviate the symptoms of cancer and thereby treating the subject.

The present method includes treating a subject suffering from a connective tissue disorder which comprises administering to the subject an amount of TGF-ß3 precursor so as to alleviate the symptoms of the disorder and thereby treating the subject.

This method further includes treating a subject suffering from a neurological disorder which comprises administering to the subject an amount of TGF-ß3 precursor so as to alleviate the symptoms of the disorder and thereby treating the subject. In one example of the invention, the neurological disorder is a demyelinating disease.

The subject method additionally includes treating a subject suffering from an immunosuppressive disorder which comprises administering to the subject an amount of TGF-ß3 precursor so as to alleviate the symptoms of the disorder and thereby treating the subject.

Additionally, this method includes treating a subject suffering from an inflammatory disorder which comprises administering to the subject an amount of TGF-ß3 precursor so as to alleviate the symptoms of the disorder and thereby treating the subject.

Further, this method includes treating a subject suffering from septic shock which comprises administering to the subject an amount of TGF-β3 precursor so as to alleviate the symptoms of septic shock and thereby treating the subject.

The present method includes treating a subject suffering from a bone disorder which comprises administering to the subject an amount of TGF-β3 precursor so as to alleviate the symptoms of the disorder and thereby treating the subject. In one example, the bone disorder is a bone fracture.

Additionally, the present method also includes treating a subject suffering from a dermatological disorder which comprises administering to the subject an amount of TGF-β3 precursor so as to alleviate the symptoms of the disorder and thereby treating the subject.

Also, this method includes treating a subject suffering from myocardial ischemia which comprises administering to the subject an amount of TGF-β3 precursor so as to alleviate the symptoms of myocardial ischemia and thereby treating the subject.

This method additionally includes treating a subject suffering from a myopathic disorder which comprises administering to the subject an amount of TGF-β3 precursor so as to alleviate the symptoms of the disorder and thereby treating the subject.

Further, this method also includes a method for treating a subject suffering from atherosclerosis which comprises administering to the subject an amount of TGF-β3 precursor so as to alleviate the symptoms of atherosclerosis and thereby treating the subject.

Additionally, this method includes a method for treating a

subject suffering from an AIDS viral infection which comprises administering to the subject an amount of TGF-ß3 precursor so as to alleviate the symptoms of the AIDS viral infection and thereby treating the subject.

The present invention also provides a method of obtaining bone marrow substantially free of actively dividing tumor cells which comprises: (a) contacting bone marrow containing normal hematopoietic cells and actively growing tumor cells with an effective amount of a TGF-ß such that the growth of the normal hematopoietic cells is temporarily inhibited; (b) subsequently contacting bone marrow with a tumor inhibiting drug under such conditions to permanently prevent growth of tumors cells; and (d) culturing bone marrow so as to permit growth of normal hematopoietic cells thereby obtaining bone marrow substantially free of actively dividing tumor cells. In accordance with the subject invention, the TGF-ß may be TGF-ß1, TGF-ß2, or TGF-ß3. TGF-ß3 is preferred.

Additionally, the present invention provides a method of obtaining bone marrow substantially free of actively dividing tumor cells. The method comprises (a) contacting bone marrow containing normal hematopoietic cells and actively growing tumor cells with an effective amount of a TGF-ß; (b) culturing bone marrow of step (a) in the presence of TGF-ß for a suitable period, under conditions such that terminal cell differentiation and clearance of tumor cells is permitted; and (e) obtaining bone marrow substantially free of actively growing tumor cells. In accordance with the claimed methods, the TGF-ß may be TGF-ß1, TGF-ß2, or TGF-ß3. TGF-ß3 is preferred.

Finally, the invention provides a method of inhibiting cytotoxic poisoning of normal cells caused by chemotherapeutic agents in which comprises contacting normal cells with an

amount of a TGF-β3 under conditions such that normal cell growth in the presence of chemotherapeutic agents is temporarily inhibited thereby inhibiting cytotoxic poisoning of normal cells.

This invention is illustrated in the Experimental Details section which follows. This section is set forth to aid an understand of the invention but is not intended to, and should not be construed to, limit in any way the invention as set forth in the claims which follow.

EXPERIMENTAL DETAILS

Abbreviations and Technical Terms:

AL (acute leukemia)
ANLL (adult non-lymphocytic leukemia)
APRT (adenosylphosphoribosyl transferase)
BFU-E (burst forming unit-erythroid)
BSA (bovine serum albumin)
CL (chronic leukemia)
CLL (chronic lymphocytic leukemia)
CML (chronic myelogenous leukemia)
CNBr (cyanogen bromide)
CFU (colony forming unit)
CFU-E (colony forming unit-erythroid)
CFU-GEMM (colony forming unit-granulocyte, erythroid, macrophage, monocyte)
CFU-GM (colony forming unit-granulocyte/macrophage)
CFU-meg (colony forming unit-megakaryocyte)
CHO (chinese hamster ovary)
CMV (cytomegalovirus)
CSF (colony stimulating factor)
DHFR (dihydrofolate reductase)
DMEM (Dulbecco's modified Eagle's medium)
DMF (dimethyl formamide)
DMSO (dimethyl sulfoxide)
DNA (deoxyribonucleic acid)
EPO (erythropoietin)
FCS (fetal calf serum)
G-CSF (granulocyte-colony stimulating factor)
GM-CSF (granulocyte/macrophage-colony stimulating factor)
kb (kilobase pairs)
kDa (kilo Daltons)
HPLC (high pressure liquid chromatography)

IL-3 (interleukin-3)
IL-4 (interleukin-4)
MEM (modified Eagle's medium)
mRNA (messenger ribonucleic acid)
RNA (ribonucleic acid)
TGF-ß (transforming growth factor-beta)
TIF (tumor inhibitory factor)
WBC (white blood cell)

EXAMPLE 1: EXPRESSION OF TGF-β3

TGF-β3 Expression Construct

A 1500 bp Alu1-Hga1 restriction fragment of TGF-β3 cDNA (sites are indicated in **Figure** 1) which encodes the complete TGF-β3 protein was cloned into the Bluescript plasmid (Strategene, La Jolla, CA) to yield the plasmid pBlue-TGF-β3. The f1 intergenic region of this vector allows the production of single stranded DNA via infection of its host bacteria with f1 helper phage. The initiation codon of TGF-β3 does not form part of a Kozak consensus sequence (CCACC[ATG]G) (11), which has been shown to influence the efficiency of translation. In order to promote high yields of the recombinant TGF-β3 protein, the flanking sequence of the initiation codon was mutagenized to a more efficient translation sequence by changing CACAC[ATG]A into CCACC[ATG]A using the method of Nakamaye and Eckstein (15). Mutagenesis was confirmed by sequence analysis. Expression yields are further optimized by deletion of TGF-ß3 5' and 3' untranslated [non-coding] sequences. Subsequently, the mutagenized pBlue-TGF-β3 was cut with KpnI and SpeI, two polylinker restriction sites flanking the cDNA insert. This fragment was cloned into the eucaryotic expression vector pORFEX (1) cut with KpnI and XbaI. In this construct (pCMV:TGF-β3) the TGF-β3 cDNA sequence is transcriptionally regulated by the cytomegalovirus immediate

early promoter (Figure 2).

DNA Transfection and Gene Amplification

Stable transformants expressing TGF-β3 were obtained by cotransfecting the pCMV-TGF-β3 construct (Figure 2) with the dihydrofolate reductase (DHFR) gene (the pDCHIP plasmid containing hamster DHFR minigene driven by its own promoter) into Chinese Hamster Ovary (CHO) cells, which lack the DHFR gene (19).

A standard $CaPO_4$.DNA precipitation method (8) was used for DNA transfection. pCMV:TGF-β3 (5.7 kb) and pDCHIP (2.5 kb) were coprecipitated with $CaPO_4$ in a ratio of 10 μg to 50ng respectively and the precipitate added to 0.5 X 10^6 CHO (DHFR-) cells. Selection of transformants with a DHFR+ phenotype was performed in alpha MEM (Gibco, Grand Island, NY) supplemented with 10% dialyzed fetal calf serum. The colonies that appeared after culturing for 10-14 days in selection medium were isolated by standard methods and expanded.

For gene amplification, the primary transfectants were subjected to stepwise selection with increasing concentrations of methotrexate (MTX; Sigma Chemical Co., St. Louis, MO). The first round of selection was carried out at 20nM MTX. TGF-β3 expression levels were measured by RNA cytodot hybridization normalizing the expression of TGF-β3 mRNA to that of actin. Two of the three clones with initial high expression (clones CHO 6.35 and CHO 9.1) showed increased TGF-β3 mRNA expression at 20nM MTX concentration (Figure 3). Total RNA (75 μg) from CHO cells (lanes 1), CHO 6.35 (lane 2), and CHO 6.35/20nM (lane 3), were fractionated on a 1.2% agarose-formaldehyde gel, blotted onto nitrocellulose and probed with a TGF-β3 specific probe (EcoRI-SmaI cDNA restriction fragment of a partial TGF-β3 cDNA clone isolated from umbilical cord; see

Figure 4). CHO 6.35/20nM (primary transfectant CHO clone 6.35 at 20nM MTX), which had the highest level of expression, was chosen for initial protein purification from conditioned media and for further gene amplification.

The best clone from further MTX selection (10 μM MTX) was expanded and a bank of frozen stocks established. This clone, 6.35H, was used in all subsequent production of TGF-β3 and was maintained in T225 flasks (225 cm^2) in alpha MEM supplemented with 10% dialyzed fetal bovine serum. TGF-β3 production involved seeding Nunc cell factories (6000 cm^2 of surface area per factory), with cells from three confluent T225 flasks of 6.35H in alpha MEM supplemented with 10% dialyzed FBS. The cells were allowed to grow to 80% confluence in the cell factories. Media was then replaced with HB CHO, a serum-free media from HANA (Hana Biologics, CA). After 72 hours, media was removed and replaced with fresh HB CHO for a total of 5 collections of conditioned HB CHO media. The first collection of conditioned HB CHO media contained low levels of TGF-β3 with the maximum amounts produced in the 4th through 6th collections. Nunc cell factories provide sufficient surface area for the large scale growth of monolayer cell lines such as CHO, yielding a total of 7.5 liters of conditioned media per factory (3 collections, 2.5 liters per collection) with acceptable ease of use in a sterile environment. Using more advanced expression vector systems, it should be possible by one skilled in the art to significantly increase production yields.

Alternatively, cell lines could be adapted to suspension growth and produced in either a stirred tank fermentation system or in an air lift fermentator. The use of porous glass cylinder supports as a means of adapting monolayer cells (i.e. CHO cells) to stirred or air-lift suspension culture has also been evaluated and shown to give acceptable yields of TGF-β3.

Expression of a mutant TGF-β3 Precursor

The mutant TGF-β3 precursor is expressed as a single homodimeric polypeptide in a host cell by mutation of the R-K-K-R cleavage site between the TGF-β3 pro region and mature TGF-β3 to a protease cleavage site, e.g. factor Xa cleavage sequence (Ile-Glu-Gly-Arg) or a collagenase cleavage sequence (Pro-X-Gly-Pro) using standard site directed mutagenesis procedures, followed by insertion of the mutant TGF-β3 nucleic acid into a expression vector and transfection of the mutant TGF-β3/vector DNA into a host cell (e.g. E. coli, CHO, or HeLa) together with DNA encoding a selectible marker (e.g. neo, dhfr).

Biological Assay for Conditioned Media

Conditioned media was treated with acetic acid to a final concentration of 0.1 M and serial dilutions tested for biological activity. CCL 64, a cell line derived from Mink lung (American Type Culture Collection, Rockville, MD), was found to be extremely sensitive to the naturally occurring TGF-β3 isolated from umbilical cord. This cell line was initially chosen, therefore, to test conditioned media for biological activity of the recombinant TGF-β3 protein according to the method of Iwata, et al. (10). Growth inhibition of CCL 64 mink lung cells produced by TGF-β1 (purified) or TGF-β3 (from conditioned media) is shown in **Figure** 5 A/B.

Figure 5A shows a dose response of growth inhibition using purified TGF-β1 (Calbiochem); a 50% inhibition was obtained with 0.1ng TGF-β1. An increase in mink cell growth inhibitory activity was found comparing conditioned media form the transfectant selected at 20nM MTX versus media from the

parental transfectant. Figure 5B shows the biological activity of acid activated serum free supernatants of CHO 6.35/20 nM transfectant (closed circles) and CHO 6.35 transfectant (open circles); 50% inhibition was obtained equivalent to 30 and 5 ng/ml TGF-β1 activity, respectively. Conditioned medium from parental CHO (DHFR-) possessed much lower growth inhibition than either transfectant. These results clearly indicate that the TGF-β3 cDNA is transcribed and that TGF-β3 mRNA is translated and produces biologically active protein.

In the presence of EGF, acidified conditioned media from CHO 6.35, containing TGF-β3 was able to promote soft agar growth of NRK cells. Growth of NRK cells in soft agar has been shown to be inducible by stimulating the production of extracellular matrix proteins, an important parameter in wound healing.

Immunodetection

Peptides corresponding to various partial amino acid sequences of the TGF-β3 protein were synthesized on an Applied Biosystems peptide synthesizer (Model 430A) using tBoc chemistry (Figure 6). Peptides were coupled to keyhole limpet hemocyanin with glutaraldehyde and used for immunization of rabbits. Enzyme-linked immunosorbent assays were used initially to characterize the antibody titers (Table 1). For this, and the following immunological experiments, standard techniques were employed (9). High titer antibody from immunized rabbits injected with β3V or β3III peptides were purified using an affinity matrix composed of the respective peptide β3 antigen conjugated to Affi-prep 10 (Bio Rad, Richmond, CA).

TABLE 1

Peptide	Sequence	Elisa Titer
I	EEMHGEREEGCTQENTESEY	1:6,000
IIL	GDILENIHEVMEIKRKGVDNEDD	1:10,000
IIs	GDILENIHEVMEIK	1:19,000
III	DTNYCFRNLEENC	1:26,000
IV	CVRPLYIDFRQDLGWKWVHEPKGYYANFC	1:19,000
V	YLRSADTTHSTVLGLYNTLNPEASASY	1:26,000
VI	CVPQDLEPLTILYYVGRTPKVEQLSNMVVKSC	1:4,000

The affinity purified β3III antibody exhibits greater than 300 fold specificity for the β3III peptide compared to the cognate peptide sequences from either the TGF-β1 or -β2. Furthermore, no significant cross reactivity of this antibody has been observed against either the TGF-β1 or -β2 proteins. However, this antibody shows only a very limited ability to immunoprecipitate the native recombinant TGF-β3 protein from conditioned media. The affinity purified β3V antibody exhibits at least a 400-fold selectively for the β3V peptide compared to the corresponding peptide sequence form TGF-β1. This antibody can also efficiently immunoprecipitate the native TGF-β3 protein (Figure 7).

Figure 8 A/B show an immunoblot of TGF-β3 in conditioned media produced by the CHO 6.35/20nM transfectant using β3III and β3V antibodies for detection. For peptide blocking experiments, the antibody was preincubated with 80-fold molar excess of peptide prior to incubation with the blot. For detection, alkaline phosphatase (Zymed, San Francisco, CA) conjugated to goat anti-rabbit IgG was used as a second antibody. Figure 8A shows a Western blot of a gel where the sample was subjected

to reduction prior to electrophoresis while Figure 8B shows the Western blot of the sample under non-reducing conditions. In each figure, lanes 1-3 and 4-6 corresponds to conditioned media immunoblotted with β3V and β3III antibody, respectively, lanes 2 and 5 immunoblots carried out in the presence of excess cognate peptide, while lanes 3 and 6 represent immunoblots in the presence of an excess unrelated peptide sequence.

Western blotting of conditioned media from CHO 6.35/20nM cells under reducing conditions, using affinity purified β3III and β3V antibody, detected a 50 kDa and a 12 kDa band. These bands correspond to the TGF-β3 precursor and mature TGF-β3, by analogy to the processing of TGF-β1 and TGF-β2 described by Gentry et al. (6) and Madisen et al. (13) (Figure 8).

Under non-reducing conditions, 100 kDa and 24 kDa bands were observed, which we believe to correspond to homodimeric forms of the TGF-β3 precursor and mature TGF-β3. The apparent precursor appears as a broad band, characteristic of some glycosylated proteins. Following cleavage of the signal peptide sequence of the precursor form of TGF-β3, one would expect a protein with MW of 43 kDa (under reduced conditions).

Based on the primary sequence of TGF-β3, there are four N-linked glycosylation sites, further indicating that the detected precursor protein is glycosylated. Figure 9 A/B show Western blot of cell extract (Figure 9A) and conditioned media (Figure 9B) of the CHO 6.35/20nM transfectant using β3V antibody for detection. For preparation of cell extracts, cells were first washed with phosphate buffered saline then lysed directly with SDS/β-mercapthoethanol prior to gel electrophoresis. For peptide blocking (lanes 2 and 4), the antibody was incubated with a 100-fold molar excess of specific peptide prior to incubation with the blot (^{125}I

protein-A was used for detection). In cell extracts of CHO 6.35/20nM under reducing conditions, only the 50 kDa band corresponding to a potential precursor form is detected (**Figure** 9 A/B). The specificity of the antibody was demonstrated by preabsorbing the antibodies with peptide immunogen prior to Western blotting (**Figures** 8 A/B and 9 A/B). As expected, based on mRNA and biological activity data, the antibody did not detect any TGF-β3 protein in conditioned media of the parental CHO (DHFR-) cells.

Both antibody were also tested for immunoprecipitation of native recombinant TGF-β3 protein (**Figure** 7). CHO 6.35/20nM were grown to confluency and labeled with [^{35}S] methionine for 24 hours in methionine-free DMEM in the presence of 5% dialyzed plus 5% non-dialyzed fetal calf serum. The medium was collected and immunoprecipitated with 10 μg/ml affinity purified antibody and 20 μg/ml (1:2 dilution) protein A agarose, for 2 hours at 4°C. Separation of the immunoprecipitated proteins on a 12.5% SDS polyacrylamide gel revealed two proteins migrating identically to the mature TGF-β3 (12 kDa) and precursor TGF-β3 (50 kDa) (**Figure** 7). However, one extra immunoprecipitated protein was found at 43 kDa.

The 43 kDa protein may correspond to either the non-glycosylated precursor or a proteolytic breakdown product. The β3V antibody, in comparison to the β3III antibody, proved to be much more efficient in immunoprecipitating the TGF-β3 protein. The specificity of the immunoprecipitation was determined by preincubating the antibody with a 80-fold molar excess of either the cognate peptide or an unrelated peptide sequence. The specific peptide showed complete competition of all three bands whereas the unrelated peptide did not. As expected, based on the amino acid composition and distribution of methionine in the TGF-β3 protein, the 50 kDa contains

significantly more ^{35}S label.

The β3V affinity purified antibody was also used in paraffin sections of human umbilical cord (**Figures** 10 A/B/C/D). Fibroblasts and epithelial cells stained (**Figure** 10A) as did the smooth muscle fibers of the cord vasculature (**Figure** 10C) whereas neither the connective tissue nor the extracellular matrix stained with this antibody. A control rabbit polyclonal antibody (Ig against P210phl/abl:OSI catalog #PC02) showed no staining (**Figures** 10 B and D). The strong staining in umbilical cord tissue agrees with earlier data showing extracts from umbilical cord possessed high levels of mRNA.

Preparation of TGF-β3 Monoclonal Antibody

A TrpE-TGF-β3 fusion was produced in E. coli which had the following characteristics, i.e. amino acids 1 to 19 are coded by the TrpE and poly linker segment and amino acids 20 to 170 correspond to amino acids 273 to 412 of the TGF-β3 precursor (containing the full mature TGF-β3 sequence). The fusion protein remained in the insoluble fraction after sonication in PBS. Subsequently, the protein was purified by separation on a SDS-polyacrylamide gel and isolated by electroelution. This material was used for immunization of mice by the following protocol:

a. Balb/C female mice were immunized intraperitoneally with 100μg of TrpE-TGF-β3 in RIBI adjuvant on days 0, 7 and 14;
b. On day 24 test bleeds indicated high titers against TrpE-TGF-β3 and purified TGF-β3 protein;
c. The mice were then boosted with 100μg of the same antigen on days 28, 29 and 30;
d. Spleen fusions were performed the following day; and

e. Subsequent methods of hybridoma selection, culture and subcloning were performed following standard procedures (9).

Five stable hybridomas were produced and their characteristics are shown in Table 2. All of the clones produced antibodies of the IgG k class. The monoclonal antibodies immunoblotted with purified TGF-β3. All five monoclonal antibodies showed no reactivity with TGF-β1 by ELISA, but crossreacted with TGF-β2.

Analysis of the epitopes recognized by the monoclonal antibodies using TGF-β3 synthetic peptides showed that all antibodies reacted with amino acids residues 380 to 412.

EXAMPLE 2: ANTIBODIES WHICH NEUTRALIZE TGF-β3 ACTIVITY

Human platelet TGF-β1 (Collaborative Research, MA), porcine TGF-β2 (R&D, Minnesota) or purified recombinant human TGF-β3, at concentrations from 3.125 to 0.049 ng/ml, was incubated with 5 μg/ml of affinity purified polyclonal rabbit antibodies (β3V antibody and anti-TGF-β (R&D, Minnesota) for 3 hours at 37°C. Control TGF-β3, TGF-β2 or TGF-β1 was incubated without antibodies. Growth inhibition of mink cells by antibody treated and control untreated TGF-β3, TGF-β2 or TGF-β1 was determined as described herein. Figures 11A, 11B and 11C show that the β3V antibody (closed squares) neutralizes the growth inhibitory activity of TGF-β3, but not TGF-β2 or TGF-β1 on mink cells relative to the growth inhibitory activity of identical concentrations of TGF-β's in the absence of antibody (open circles). Anti-TGF-β (R&D, Minnesota) neutralizes TGF-β3, TGF-β2 and TGF-β1 (Figures 11A, 11B and 11C)(closed circles). Neither antibody had any significant effect on the growth of CCL-64 cells in the absence of TGF-β3. Antibodies against the TGF-β3 peptide β3V apparently specifically

neutralizes the growth inhibitory activity of TGF-β3.

EXAMPLE 3: EVALUATION OF ANTI-CANCER ACTIVITY IN VITRO

Growth was determined using a modification of the monolayer assay for TGF-β3 described by Iwata, K.K., et al. (10). Non-leukemic cells were subcultured on 96-well tissue culture plates in 100μl of media at a seeding density of $2x10^3$ cells per well. Cells were maintained and assayed in Dulbecco's modified Eagle's medium containing 10% fetal bovine and 2% L-glutamine. These cells were treated with 25 ng/ml (~1nM) of TGF-β3, pulsed 24 hours with 1μCi/ml 5-[^{125}I]-iodo-2'deoxyuridine when cells in the untreated control wells were 90% confluent and harvested.

Leukemic cells (K562, KG-1, KG-1a, HuT 78 and U937) were seeded in 50μl of media. K562 was seeded at a density of $1x10^3$ cells per well in RPMI supplemented with 10% fetal bovine serum. KG-1 and KG-1a were seeded at a density of $3.5x10^3$ cells per well in Iscove's media supplemented with 10% fetal bovine serum. Hut 78 and U937 were seeded at a density of $3.5x10^3$ cells per cell in RPMI supplemented with 10% fetal bovine serum. Cell growth was determined by microscopic examination. Examples are shown in Table 2, showing inhibition of some human tumor lines by TGF-β3.

EXAMPLE 4: DEVELOPMENT OF ANTIGEN CAPTURE ASSAY FOR TGF-β3

Plates are coated with 50 μl of affinity-purified rabbit polyclonal antibody (5μg/ml in 0.1M $NaHCO_3$, pH 9.1) made to TGF-β3 peptide β3V. Plates were incubated overnight at 4°C. Unbound antibody is removed by aspiration. Plates are blocked with 100 μl PBS containing 1% BSA (PBS-BSA) for 1 hr at room temperature. The plates are then washed twice with phosphate-buffered saline (PBS) containing 0.05% Tween 20 (PBST).

Samples in a final volume of 50 µl of PBS-BSA are added to the appropriate wells and incubated for 1 hr at room temperature. Unbound protein is removed and the plate is washed four times with PBST. All wells receive 50 µl of mouse monoclonal antibody against TGF-β3 (5µg/ml in PBS). After incubation for 1 hr at room temperature, unbound antibody is removed and the plate is washed four times with PBST. All wells receive 50 µl of an appropriate dilution of alkaline phosphatase conjugated to goat anti-mouse antibody. After incubation for 1 hr at room temperature, the plate was washed four times with PBST.

Substrate for alkaline phosphatase (5-bromo-4-chloro-3-indolyl phosphate) in 100 µl is added to all of the wells and incubated for 15 min at room temperature. Absorbance in each well measured at 490 nm. Using this assay, we detected between 3-5ng/ml recombinant TGF-β3.

EXAMPLE 5: USE OF TGF-β3 IN BONE DISORDERS

TGF-β like activities are produced by bone cells and large amounts are found in the extracellular bone matrix, indicating an important physiological function of TGF-βs in this tissue (2). TGF-β stimulates cell replication and collagen production in cultured fetal rat bone cells (3, 4, 5) and induces chondrogenesis of embryonic rat mesenchymal cells (17). In addition, molecules with TGF-β like activity are released in vitro after bone resorption and may effect a link between the coupled processes of bone formation and resorption during remodeling (16, 18).

TABLE 2

Effects of TGF-β3 (1nM) on the Growth of Human Cell Lines in Culture

CELL LINE		% INHIBITION
Human Tumor		
A549	(lung adenocarcinoma)	46
A375	(melanoma)	47
A2058	(melanoma)	88
WiDR	(colon adenocarcinoma)	24
MCF 7	(breast carcinoma)	57
Human Leukemic Cells		
K562	(CML)	55
KG-1	(AML)	50
KG-1a	(AML)	50
HuT 78	(T cell lymphoma)	50
U937	(histiocytic lymphoma)	50
Normal Human		
Huf	(foreskin fibroblasts)	6

The experimental paradigm is to isolate various cell populations from resected bone fragments by sequential collagenase digestions (3, 4, 5). The later released populations are enriched for bone forming cells with the biochemical characteristics associated with the osteoblast phenotype, such as type I collagen production, elevated alkaline phosphatase activity and osteocalcin synthesis (14). Studies with such isolated bone cells have shown that TGF-β1 is a potent regulator of cells from the osteoblast lineage (4). On a molar basis, TGF-β1 is one of the most potent mitogens thus far described for osteoblast-enriched cultures from fetal bone. The mitotic response to TGF-β1 is biphasic with an optimal concentration below 100pM (2, 3, 4). TGF-βs, in addition, alter expression of various activities associated with the osteoblast phenotype: alkaline phosphatase activity is decreased while the synthesis of type I collagen is enhanced similar to the effects of TGF-β in a number of other connective tissue systems (3, 4).

The experiments described below were performed to assess the effects of human recombinant TGF-β3 on osteoblast-enriched cultures from fetal rat parietal bone and to characterize the specific binding of TGF-β3 to bone cell-surface proteins.

Cell cultures

Parietal bones dissected free of adjacent suture lines were obtained from 22 day old rat fetuses (Sprague-Dawley, Charles River Breeding Laboratories, Wilmington, MA) and were subjected to 5 sequential 20 min collagenase digestions as previously described (14, 20). The population of cells released during the first enzyme treatment (population 1) is enriched with less differentiated fibroblast-like cells,

whereas the last three populations (numbers 3-5) are enriched with cells expressing features characteristic of the osteoblast phenotype.

Cells from population 1 and a pool of cells from populations 3-5 were plated at 12,500 cells/cm^2 in 0.32 cm^2 wells and were cultured in Dulbecco's modified Eagle's medium as has been detailed (3, 4, 14). After reaching confluency (approximately 6-8 x 10^4 cells/cm^2), the cultures were deprived of serum for 20 hrs; the factors of interest were then added to the cultures in serum-free medium and incubated for an additional 23 hours.

DNA synthesis

To examine the mitogenic effect of the test factors, cell cultures were pulse labeled with [^{3}H]thymidine (80Ci/mmol) for the last 2 hours of treatment and lysed by the addition of 0.1M sodium dodecyl sulfate and 0.1N sodium hydroxide. The insoluble material formed by precipitation with 10% TCA was collected on glass fiber filters, rinsed with ethanol and measured by scintillation counting. Data are shown as the total amount of acid-precipitable [^{3}H]thymidine incorporated per 0.32 cm^2.

Protein synthesis

To measure collagen and noncollagen protein synthesis, 2 cm^2 cultures were pulsed with 12.5μC/ml [^{3}H]proline (125 mCi/mmol) for the last 2 hours of culture. Cells were rinsed with isotonic buffer (146 mM NaCl, 11 mM dextrose, 35 mM Tris-HCl, pH 7.4) and lysed by freeze-thawing in 0.5% (v/v) Triton X-100 (Sigma). The homogenates were diluted 3-fold, precipitated with 10% trichloroacetic acid, and the acid-precipitable material collected by centrifugation. The pellets were

acetone-extracted, dried, resolubilized in 0.5 M acetic acid and neutralized with NaOH. The amount of [^{3}H]proline incorporated into collagenase-digestible protein and noncollagen protein was measured as described (23).

Alkaline phosphatase assay

Enzyme activity was measured in extracts prepared from 2 cm^2 cultures following sonication in 0.5% Triton X-100. Hydrolysis of p-nitrophenyl phosphate was measured at 410 nm after 30 min (12); data are expressed as nanomoles of p-nitrophenol released per min/mg protein.

Biological activity of TGF-β3 on osteoblasts

TGF-β like activities are produced by bone cells and large amounts are found in the extracellular bone matrix, indicating an important physiological function of TGF-βs in this tissue (2). TGF-β stimulates cell replication and collagen production in cultured fetal rat bone cells (3, 4, 5) and induces chondrogenesis of embryonic rat mesenchymal cells. In addition, molecules with TGF-β like activity are released in vitro after bone resorption and may effect a link between the coupled processes of bone formation and resorption during remodeling.

In this example, we isolated various cell populations from resected bone fragments by sequential collagenase digestions (2, 3) releasing populations enriched for bone forming cells with the biochemical characteristics associated with the osteoblast phenotype, such as type I collagen production, elevated alkaline phosphatase activity and osteocalcin synthesis (Figure 12 A, B, C).

Recombinant TGF-β3 bound to specific receptors and had a

biphasic stimulatory effect of DNA synthesis, enhanced collagen synthesis and decreased alkaline phosphatase activity in osteoblast-enriched cultures after 23 hours of treatment as shown in Figure 12 A, B, C. When protein concentration of TGF-β3 and TGF-β1 were normalized using both the colloidal gold assay from Collaborative Research (Bedford, MA) and silver staining on an SDS polyacrylamide gel, TGF-β3 was significantly more potent than TGF-β1, with an approximate 3-5 fold lower concentration needed for similar half maximal effects in all three of the above-described biological activities. These results show TGF-β3 to be a potent stimulator of bone cell growth and function.

EXAMPLE 6: EFFECTS OF TGF-β ON THE CELLS OF THE CENTRAL AND PERIPHERAL NERVOUS SYSTEM

TGF-β3's utility extends to the repair of neurological disorders by accelerating regeneration of peripheral nerves and in promoting regenerative phenomena in the central nervous system. TGF-β1 appears to stimulate the DNA synthesis of short-term Schwann cell (50). In contrast to its effect on short-term Schwann cells, TGF-β1 inhibited DNA synthesis in glial cells and in long term Schwann cells. Utility of TGF-β3 extends to controlling glial proliferation during development and/or regeneration of the peripheral nervous system. Immunohistochemical staining indicates that TGF-β3 is selectively expressed in the mammalian nervous system (51).

EXAMPLE 7: EFFECTS OF TGF-β ON MUSCLE CELLS

TGF-β1 is a potent modulator of myocyte differentiation. TGF-β1 blocks the onset of differentiation when added to undifferentiated myoblasts and causes differentiation when added to fully differentiated myocytes (53). TGF-β1 inhibits proliferation (53) and DNA synthesis of myocytes. TGF-β3 is

involved in the regulation of cardiomyocyte proliferation. TGF-β1 mRNA and protein is rapidly lost following myocardial infarction caused by ligation of a coronary artery (53). After the ligation, there is a marked increase in the TGF-β1 mRNA indicating a significant role for TGF-β in response to the injury to the heart. TGF-β2 and TGF-β3 mRNAs were all expressed constitutively in cultured myocytes extracted from embryonic hearts. TGF-β3 is expressed in skeletal and cardiac myocytes (52). Accordingly, this data extends TGF-β3's therapeutic utility to the repair of muscle after cardiac injury. Moreover, antibodies directed against TGF-β3 and the pro region of TGF-β3 may have a similar therapeutic utility in modulating the activity of TGF-β3 in the repair of muscle after cardiac injury.

EXAMPLE 8: EFFECTS OF TGF-β3 IN ENDOTHELIAL CELLS

TGF-β3 is a potent inhibitor of endothelial cells. Endothelial cell growth is implicated in the control of neovascularization and plaque formation in atherosclerosis. Accordingly, this data extends the utility of TGF-β3 on modulating dysfunctions involving endothelial cell proliferation including atherosclerosis.

EXAMPLE 9: EVALUATION OF TGF-β3 FOR IMMUNOSUPPRESSIVE ACTIVITY IN VITRO

Lymphocytes were separated from whole blood using Ficoll-Paque (Pharmacia LKB Biotechnology Inc.). Separated lymphocytes were cultured in T25 flasks in RPMI media containing 10% FCS and IL2 (Lymphocult-Biotest Diagnostics). The cells were maintained in this media to grow out the T-lymphocytes. Actively growing T-cells (2.2×10^5 cells/ml) in 10 ml of media were placed in T25 flasks with and without 8 ng/ml TGF-β3. Cell growth was determined by microscopic examination at

40 x magnification and quantitation using a Coulter counter. After 5 days, it was observed that untreated T cells formed a large number of aggregates (~16 in a field of 0.2 cm^2), presumably as single cell divide multiple times without separating. Coulter counter quantitation showed 4.15 x 10^5 cells/ml. Cells treated with TGF-β3 at 8 ng/ml formed very few small aggregates and were 3.2 x 10^5 cells/ml. TGF-β3 inhibited the proliferation of fresh human T cells showing that it is immunosuppressive. The effects of TGF-β3 on cells mediating immune and inflammatory responses indicates TGF-β3's utility in controlling disorder such as immune dysfunction, inflammation, and septic shock.

EXAMPLE 10: USE OF TGF-β3 AS A CHEMOPROTECTION AGENT

The rationale for these experiments is to define conditions which allow for use of TGF-β3 in vivo to protect the immune system during chemotherapy thus preventing infection and additionally allowing use of higher doses of chemotherapy in treatment.

Chemoprotection in vitro

Primary human bone marrow and peripheral blood samples are evaluated for the growth of the different hematopoietic lineages in the presence and absence of exogenous TGF-β3. Stem cell cultures are purged of mature, well differentiated cells. Specifically, buffy coat cells is collected by centrifugation (800rpm, 10', 5°C), suspended in McCoy's medium including 10% heat inactivated FCS ('complete medium'). Platelets are removed by Percoll gradient centrifugation (1.050g/ml; Pharmacia) and a low density, small primitive cell population obtained after re-centrifugation on a Percoll gradient (1.075g/ml). Individual populations of B- and T-lymphocytes, granulocytes, monocytes and more differentiated

erythroid populations can be immunodepleted (5) by panning $2x10^8$ light-density cells with monoclonal antibodies (anti-B1, anti-B4, anti-LyT3, anti-My4, anti-My8, anti-903, anti-N901, anti-Leu1 and anti-glycophorin A (R10) and WEM-G11) directed against mature cell surface epitopes for 30 minutes on ice. Cells are washed twice in cold complete medium and assayed for progenitor cells (48, 49). The primitive stem cell population are grown in methylcellulose support (Iscove's modified Dulbecco's medium (IMDM; Gibco), 24% FCS, 0.8% dialyzed bovine serum albumin, 100μM β-mercaptoethanol, and 1.3% methylcellulose in 35mm Lux culture plates) in the presence of 10pM, 100pM and 1000pM TGF-β3 with and without Mo T-lymphocyte conditioned media, a source of colony stimulating factors (44) in quadruplicate cultures maintained in 5% CO_2.

Colony forming units of the various hematopoietic lineages are counted microscopically at 3, 7, 14 and 21 days. An example of this experiment is shown in **Figure** 13. Individual colonies may be aspirated onto glass slides, selectively stained with May-Grunewald-Giemsa and the presence of neutrophils, monocytes or eosinophils observed. These experiments allow one skilled in the art to determine if the growth inhibitory action of TGF-β3 is lineage-specific at given doses of TGF-β3, determine the time course of inhibition and determine the dose of TGF-β3 required for inhibition of a given cell type.

As a general rule, the smaller hematopoietic precursors represent more primitive progenitor stem cells while the larger cells are usually more mature, as analyzed by the appearance of maturation-specific cell surface epitopes. Enriched progenitor populations obtained by immuno-depletion as described may be size selected by Percoll gradient centrifugation and different size cell populations evaluated for specific lineages in combinations of Mo conditioned media and TGF-β3 at 3, 7, 14 and 21 days. Assays for early stem

cell populations (HPP-CFU of CFU-A), progenitor cells (CFU-E, CFU-GEMM, BFU-E, CFU-MK), pre-B colony, B colony, T colony, cytolytic T cell, and antigen stimulation assays are currently well developed.

Colonic epithelium

The growth inhibitory effects of TGF-β3 on primary normal and neoplastic colon organ cultures may be evaluated with a view to establishing TGF-β3 as a chemoprotective agent to reduce gastrointestinal toxicity in the treatment of patients with chemotherapeutic drugs.

Proliferation of colon cell in various stages of differentiation is measured by [^{3}H]-thymidine incorporation into colon biopsy organ cultures followed by sectioning and staining of the intact crypt. A 1mm biopsy specimen is gently washed in DMEM, 10% FCS, 37°C. Three micron sections are cut to avoid unequal distribution of radiolabel. Preincubation with TGF-β3 at 10pM, 100pM, and 1nM is carried out over several time points.

Colon specimens are labeled in 2ml of DMEM + 10%, 37°C equilibrated in a 5% CO_2 incubator, containing 1μCi/ml [^{3}H]-thymidine (20Ci/mmole) for 1 hours. Sections are washed, fixed in 10% formalin, embedded and cut longitudinally to expose the morphology of the colon crypt. Tissue sections are coated with liquid emulsion and autoradiographed.

The proliferation index of cells in various stages of crypt development is determined by microscopic counting of exposed silver grains. Routinely, cells with >4 grains score as positive. In normal tissue, only the lower third of the crypt (containing the stem cell population) are labeled. Adjacent tissue serves as an internal control. The appearance of

differentiation markers on colon crypt cells are monitored using available monoclonal antibodies to cytokeritins and colon specific antigen (fetal).

To establish in vitro models for the chemoprotective effects of TGF-β3, doses of cytotoxic drug in organ culture or in dispersed mixed cell culture required for toxicity are assessed. Organ cultures are prepared as previously described (45). Parallel cultures are incubated in a range of 5-FU concentrations and proliferation measured by [^{3}H]-thymidine incorporation and sectional autoradiography. To establish dispersed, mixed cell colon cultures, biopsy material are cut to >0.5 cm^2, washed in phosphate buffered saline (PBS), finely titrated, centrifuged, rinsed, washed (5X) and cultured in a mixture of Leibowitz's medium L15 and suspension modified MEM (SMEM) with a final Ca^{+2} concentration of 0.5mM, 10% fetal calf serum, 100 units penicillin, 50μg/ml streptomycin, 25μg/ml gentamicin, 2mM glutamine, 1ng/ml epidermal growth factor (EGF), 20μg/ml insulin, 10μg/ml transferrin, 25nM sodium selenite and grown on collagen (Type I) coated culture plates (46, 47).

Colon cells grown on collagen coated coverslips in a range of 5-FU concentrations are incubated in [^{3}H]-thymidine (0.2μCi/ml) for thirty minutes, washed and chased overnight in fresh media. Cells are washed, fixed/stained, coverslips dipped in liquid photographic emulsion and autoradiographed. Proliferation is measured by counting exposed silver grains.

Chemoprotection by TGF-β3 in vitro is measured by pre-addition of a range of TGF-β3 doses to colon organ or cell cultures followed by 5-FU at or near the toxic dose, and [^{3}H]-thymidine labelling at intervals after exposure to cytotoxic drug as a measure of cellular recovery.

TGF-β3 acting as a chemoprotectant would inhibit the high proliferative growth rate of the normal bone marrow and the intestinal crypt cells. Therefore, TGF-β3 would decrease the life-threatening side effects of conventional chemotherapy and allows the use of more aggressive dose regimens and maintain colonic integrity and prevent infection.

Chemoprotection in vitro

In order to evaluate TGF-β3 as a chemoprotective agent in vitro the following experiment was performed.

Mink cells were seeded in 96-well plates at 10^3 cells/well in 100μl of DMEM supplemented with 10% fetal bovine serum. Wells containing treated cells received 25μl of TGF-β3 (50ng/ml). After 24 hours incubation with TGF-β3, 25μl of colchicine or vinblastine was added. After another 24 hours, the media was removed and the cells washed once with Dulbecco's PBS and fresh complete media added. The cells were incubated for another 7 days.

Cell growth was quantitated by uptake of 5-[^{125}I]iodo-2'deoxyuridine (^{125}IUdR) indicating the amount of cell growth as previously described. As seen in Figure 14, cells preincubated with TGF-β3 prior to incubation with various doses of chemotherapeutic drugs (i.e. vinblastine and colchicine) showed significantly more uptake of ^{125}UIdR relative to cells which were incubated with the chemotherapeutic drugs without TGF-β3. Therefore, cells preincubated with TGF-β3 were protected from the toxic effects of the chemotherapeutic drugs. Similar results were observed when adriamycin was used as the chemotherapeutics drug. This chemoprotective effect should also be possible with other chemotherapeutic drugs including, but not limited to, 5-fluorouracil, and etoposide.

For TGF-β3 to be an effective chemoprotectant, it is apparent that the growth of the tumor must be less inhibited than that of normal tissues. This may be achieved either because the tumor is innately resistant to the growth inhibitory effects of TGF-β3 or via the pharmacokinetics allowing a differential effect in vivo.

The following experiments detail steps used to demonstrate efficacy of TGF-β3 for chemoprotection in vivo.

Acute Toxicology and Chemoprotection of Normal Mice

Acute administration of escalating doses of TGF-β3 can be investigated for toxicity (survival, weight loss) in normal Balb/c mice. Various hematologic parameters, progenitor cell assays and immune function assays are undertaken.

The status of the hematopoietic stem cell compartment (total numbers in marrow, spleen and circulation, and cell cycle status) is determined using the in vivo CFU-S assay, and the in vitro CFU-GEMM and high proliferative potential (HPP) CFU assays. Progenitor cells for the erythroid (BFU-E, CFU-E), myeloid (CFU-GM, CFU-M, CFU-G) and megakaryocyte/platelet series (CFU-MK) are assayed. Lymphoid function is measured by B- and pre B-lymphocyte colony assays, etc. Subsets of myeloid and lymphoid cells in tissues is determined by FACS analysis using lineage-specific MAbs. WBC, platelets, red blood count (RBC) and hematocrit are measured in repeated tail vein blood samples. Serum samples are obtained and are assayed for TGF-β, G-CSF, GM-CSF, M-CSF, IL-1,3,4,5 and TNF by bioassay and radioimmunoassay. Neutrophil function assays include in vivo and in vitro chemotaxis, bactericidal capacity and receptor expression for multiple cytokines.

The ability of TGF-β3 to protect hematopoietic stem cells from

the cytotoxic effects of chemotherapeutic agents is assessed in animal models. Untreated BALB/c mice, or groups pre-treated with TGF-β3, receive a single i.v. dose of 5-FU (150mg/kg). In subsequent experiments cyclophosphamide (200mg/kg), vinblastine (2.5mg/kg), adriamycin (2.5mg/kg) or methotrexate (150mg/kg) is employed. The number of peripheral circulating blood cells and the various hematopoietic progenitor cells are determined, including CFU-S, CFU-GM, HPP-CFU-C, BFU-E, CFU-Mk and CFU-GEMM.

Experiments include altering the dose of TGF-β3 (0.1, 0.5, 2.0, 5.0 and 10.0 μg/animal) as well as the time course of TGF-β3 administration with respect to chemotherapy (48h, 24h or 12h before chemotherapy).

Pharmacokinetics of TGF-β3

The chemical half-life of TGF-β3 is determined in the serum of mice following bolus injections (0.1-10μg/mouse) via i.v., i.p., and s.c. routes, using internally labelled TGF-β3 (labelled metabolically with ^{35}S cysteine) or ^{125}I TGF-β3. Tissue distribution of labelled material is measured in various organs with particular emphasis on liver, spleen and bone marrow sites. If the biological half-life of TGF-β3 in vivo is found to be unacceptably short regional administration by direct intrasplenic injection (through the body wall) is employed or using the surgical technique reported by Goey et al (7) involving injection into the femoral artery. This latter approach has been reported to be effective in localizing TGF-β1 to the marrow with resulting inhibition of early stem cell and progenitor cell proliferation.

Chemotherapy and TGF-β3 in a Spontaneous Breast Tumor Model

The translation of preclinical laboratory results to clinical

cancer therapy depends to a very large extent on the relevance of the laboratory model employed. Because of the obvious shortcomings of the long transplanted murine tumor models and the xenograft human tumor models in nude mice, it is preferable to select the CDF1 breast tumor model. CDF1 shows a remarkable 100% correlation in chemotherapeutic sensitivity to drugs which are considered to be active against human breast cancer.

It is preferable, although not necessary, to identify the optimal parameters of dosage and scheduling relationships by the experiments hereinabove, i.e. TGF-β3 is tested in this model. Most of the planned studies are performed using first generation syngeneic transplants of spontaneous breast tumors. In addition to determination of life span, the effect of therapy is determined on tumor growth rate, on partial and complete regression, and on spontaneous metastasis (determined histologically or by tissue retransplantation). TGF-β3 is tested for its ability to mediate a reversible cytostatic block on hematopoietic progenitor and stem cell proliferation, conferring resistance to toxicity of chemotherapy.

These experiments define conditions in which TGF-β3 can act as a chemoprotective agent in vivo. These conditions are used by one skilled in the art to administer to a patient a suitable amount of TGF-β3 prior to cytotoxic chemotherapy.

EXAMPLE 11: USE OF TGF-β3 IN AUTOLOGOUS BONE MARROW TRANSPLANTATION

Autologous bone marrow transplantation is a method in which the bone marrow of a patient is removed prior to chemotherapy to reduce hematopoietic stem cell toxicity. Autologous bone marrow transplantation has also been performed in patients with acute nonlymphocytic leukemia using 4-

hydroperoxycyclophosphamide to treat the marrow ex vivo (21).

In one instance, TGF-β3 is used to inhibit the proliferation of the bone marrow stem cell population prior to chemotherapy of bone marrow in vivo in patients with blood-bone tumor cells. Briefly, TGF-β3 is contacted with the patient bone marrow at a sufficient concentration to inhibit the normal hematopoietic cells (for example, 1-1000 pM) as determined by one skilled in the art. At a given time after TGF-β3 treatment of bone marrow ex vivo (typically but not limited to 6-24 hours or as determined by the physician) and the tumor cell population refractory to the effects of TGF-β3, the cells are treated with a cytotoxic chemotherapeutic agent (e.g. adriamycin, 5-fluorouracil, and vinblastine). The treated marrow is returned to the patient at a time determined by the physician and the normal hematopoietic cells allowed to recover from the growth inhibiting effects of TGF-β3 and proliferate, thus reconstituting that component of the patients hematopoietic system.

Alternatively, the bone marrow cells are treated with TGF-β3 as described and the bone marrow cells cultured ex vivo as described (118) such that the leukemic cell population continues to proliferate and terminally differentiate while the normal population is growth arrested. Continued culture in this way results in the terminal growth arrest of the leukemic population and enrichment of the normal cell population. Further, enrichment of the normal cell population may be accelerated by contacting the normal cell population with hematopoietic growth factors, e.g. GM-CSF and IL-3. Bone marrow thus treated is returned to the patient, substantially free of leukemic cells.

Example 12: Expression of a Pro Region of the TGF-β3 Precursor Separated from Mature TGF-β3

The pro region of the TGF-β3 precursor associates with the mature TGF-β3 and modifies the biological activity and half life of mature TGF-β3. Nucleic acid encoding the TGF-β3 precursor beginning with methionine at nucleotide positions 263-265 and ending with arginine at position 1160-1162 is engineered by mutagenesis of the nucleic acid in Figure 1 to introduce a translation termination codon (TGA, TAG, TAA) at position 1163-1165. The resulting nucleic acid is inserted in an expression vector and transfected into a suitable host cell with an additional selectible marker, as previously described. TGF-β3 pro region protein is recovered from the culture medium. This protein binds mature TGF-β3 and thereby sequesters and modifies the half life and biological activity of the mature TGF-β3.

Binding assay of TGF-β3 to the TGF-β3 pro region protein

The binding of TGF-β3 pro region to TGF-β3 or mutant TGF-β3 is measured by the following. ^{125}I TGF-β3 is incubated with purified TGF-β3 pro region in PBS with either unlabeled TGF-β3 or mutant TGF-β3 for 5 hr on ice. Non-specific binding is determined using a 400-fold molar excess of unlabeled growth factor. Crosslinking of the TGF-β3 pro region to ^{125}I TGF-β3 is accomplished with the addition of a ¼ volume of 5 mM bis(sulfosuccinimidyl)suberate (BS^3; Pierce) in PBS and the reaction is stopped after 2 min at 4°C by the addition of 1/20 volume of 2.5 M glycine. An equal volume of SDS-PAGE sample buffer (2X) is added to the sample is heated in a boiling water bath for 3 min.

Electrophoresis is performed on the samples and destained gels

is dried and exposed at -70°C to X-ray film using intensifying screens. Alternatively, antI-TGF-β3 is used to immunoprecipitate the TGF-β3 pro region complexed with ^{125}I TGF-β complex with or without crosslinking and quantitated directly by a gamma counter.

Example 13: Inhibition of TGF-β by a TGF-β binding protein

It would be clear to those skilled in the art that TGF-β3 is a bifunctional growth factor. The experiments disclosed herein illustrate that TGF-β3 inhibits or stimulates the same target cell depending upon the exposure time and concentration of other exogenous factors. As a potent modulator of cell growth and differentiation, the regulation of TGF-β3 levels and exposure of target cells to TGF-β3 in concert with these other factors is important for normal tissue function and development.

Specifically herein the pro region of the TGF-β precursor and the antibodies directed against the mature TGF-β can be administered to a patient in a suitable carrier in a pharmaceutically suitable amount to neutralize, or modify clearance of, systemic TGF-β thereby treating the patient suffering from a disorder or symptoms associated with excess TGF-β. Examples of disorders associated with excess TGF-β include, but are not limited, connective tissue disorder (for example fibrosis and scleroderma), immunosuppression, myocardial ischemia, myopathic disorder, certain cancers associated with elevated levels of TGF-β (for example glioblastoma), neurological, inflammatory, AIDS viral infection and atherosclerosis.

Additionally, the pro region of the TGF-β precursor could be used as a vehicle for delivery of mature TGF-β thereby modifying the half life and biological activity of the mature

TGF-β.

Example 14: Effects of TGF-β3 on Fibroblasts

TGF-β3 enhances cell growth, alone or in combination with other molecules. For example, TGF-β3 may directly affect DNA synthesis. Alternatively, TGF-β3 synergizes with other factors to promote cell growth. Accordingly, when contacted with fibroblasts *in vitro* or *in vivo*, TGF-β3 acts to promote connective tissue repair, dermatological repair and wound healing.

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What is claimed is:

1. An antibody which (a) specifically binds to mature TGF-β3 and (b) exhibits substantially no cross reactivity with TGF-β1 or TGF-β2.

2. The antibody of claim 1, wherein the antibody is directed to an epitope defined by the amino acid sequence DTNYCFRNLEENC.

3. The antibody of claim 1, wherein the antibody is directed to an epitope defined by the amino acid sequence YLRSADTTHSTVLGLYNTLNPEASASY.

4. The antibody of any of claims 1, 2, or 3, wherein the antibody is a monoclonal antibody.

5. The antibody of any of claims 1, 2, or 3, wherein the antibody is a polyclonal antibody.

6. An antibody which (a) specifically binds to a pro region of TGF-β3 precursor and (b) exhibits substantially no cross reactivity with mature TGF-β3.

7. A method of detecting a TGF-β3 precursor from a sample which comprises contacting the sample with a suitable amount of the antibody of claim 6, under conditions such that the antibody binds to the TGF-β3 precursor and detecting the antibody bound to the TGF-β3 precursor and thereby detecting the TGF-β3 precursor from the sample.

8. A method of detecting a pro region of a TGF-β3 precursor from a sample which comprises contacting the sample with a suitable amount of the antibody of claim 6, under

conditions such that the antibody binds to the pro region of the TGF-β3 precursor and detecting the antibody bound to the pro region of the TGF-β3 precursor and thereby detecting the pro region of the TGF-β3 precursor from the sample.

9. A method of diagnosing a disorder associated with a variation in TGF-β levels in a human subject which comprises (1) obtaining a sample from the subject, (2) detecting the presence of a TGF-β in the sample, and (3) determining the amount of TGF-β thereof in the sample thereby diagnosing the disorder.

10. The method of claim 9, wherein the disorder is osteoporosis.

11. The method of claim 9, wherein the disorder is an immune-suppressive disease.

12. The method of claim 9, wherein the disorder is an AIDS viral infection.

13. The method of claim 9, wherein the disorder is a dermatological disorder.

14. The method of claim 9, wherein the disorder is myocardial ischemia.

15. The method of claim 9, wherein the disorder is a myopathic disorder.

16. The method of claim 9, wherein the disorder is a connective tissue disorder.

17. The method of claim 9, wherein the disorder is a

neurological disorder.

18. The method of claim 9 wherein the TGF-β is TGF-β1.

19. The method of claim 9, wherein the TGF-β is TGF-β2.

20. The method of claim 9, wherein the TGF-β is TGF-β3.

21. The method of claim 9, wherein the variation in TGF-β levels is a variation in mature TGF-β3 levels and detection is effected by an antibody which specifically binds to mature TGF-β3 and does not exhibit cross reactivity with mature TGF-β1 or mature TGF-β2.

22. The method of claim 9, wherein the variation in TGF-β levels is a variation in mature TGF-β3 levels and detection is effected by an antibody which specifically binds to the pro region of TGF-β3 and exhibits substantially no cross reactivity with mature TGF-β3.

23. A method for treating a subject suffering from a disorder associated with a TGF-β which comprises administering to the subject an amount of an antibody which specifically recognizes TGF-β and neutralizes TGF-β activity.

24. The method of claim 23, wherein the disorder is a cancer.

25. The method of claim 23, wherein the disorder is arthritis.

26. The method of claim 23, wherein the disorder is an immunosuppressive disease.

27. The method of claim 23, wherein the disorder is an AIDS viral infection.

28. The method of claim 23, wherein the disorder is myocardial ischemia.

29. The method of claim 23, wherein the disorder is a myopathic disorder.

30. The method for of claim 23, wherein the disorder is a connective tissue disorder.

31. The method for of claim 23, wherein the disorder is a atherosclerosis.

32. The method for of claim 23, wherein the disorder is a neurological disorder.

33. The method for of claim 23, wherein the disorder is a bone disorder.

34. The method of claim 23, wherein the antibody specifically recognizes mature TGF-β and exhibits substantially no cross reactivity with the pro region of the TGF-β precursor.

35. The method of claim 23, wherein the antibody specifically binds to mature TGF-β3 and exhibits substantially no cross reactivity with mature TGF-β1 or mature TGF-β2.

36. The method of claim 23, wherein the antibody is a humanized antibody.

37. The method of claim 23, wherein the antibody is a F(ab) fragment.

38. The method of claims 23, wherein the antibody is a

F(ab')2 fragment.

39. The method of claim 23, wherein the antibody is a monoclonal antibody.

40. The method of claim 23, wherein the antibody is a polyclonal antibody.

41. A pharmaceutical composition comprising an effective amount of a pro region of a TGF-β precursor and a suitable pharmaceutical carrier.

42. The composition of claim 41, wherein the pro region of the TGF-β precursor is the pro region of the TGF-β1 precursor.

43. The composition of claim 41, wherein the pro region of the TGF-β precursor is the pro region of the TGF-β2 precursor.

44. The composition of claim 41, wherein the pro region of the TGF-β precursor is the pro region of the TGF-β3 precursor.

45. A method for treating a subject suffering from a cancer which comprises administering to the subject an amount of the pharmaceutical composition of claim 41 so as to alleviate the symptoms of cancer and thereby treating the subject.

46. A method for treating a subject suffering from a connective tissue disorder which comprises administering to the subject an amount of the pharmaceutical composition of claim 41 so as to alleviate the symptoms of the disorder and thereby treating the subject.

47. A method for treating a subject suffering from a neurological disorder which comprises administering to the subject an amount of the pharmaceutical composition of claim 41 so as to alleviate the symptoms of the disorder and thereby treating the subject.

48. A method for treating a subject suffering from an immunosuppressive disorder which comprises administering to the subject an amount of the pharmaceutical composition of claim 41 so as to alleviate the symptoms of the disorder and thereby treating the subject.

49. A method for treating a subject suffering from a bone disorder associated with a TGF-β which comprises administering to the subject an amount of the pharmaceutical composition of claim 41 so as to alleviate the symptoms of the disorder and thereby treating the subject.

50. A method for treating a subject suffering from myocardial ischemia which comprises administering to the subject an amount of the pharmaceutical composition of claim 41 so as to alleviate the symptoms of myocardial ischemia and thereby treating the subject.

51. A method for treating a subject suffering from a myopathic disorder which comprises administering to the subject an amount of the pharmaceutical composition of claim 41 so as to alleviate the symptoms of the disorder and thereby treating the subject.

52. A method for treating a subject suffering from atherosclerosis which comprises administering to the subject an amount of the pharmaceutical composition of

claim 41 so as to alleviate the symptoms of atherosclerosis and thereby treating the subject.

53. A method for treating a subject suffering from arthritis which comprises administering to the subject an amount of the pharmaceutical composition of claim 41 so as to alleviate the symptoms of arthritis and thereby treating the subject.

54. A method for treating a subject suffering from an AIDS viral infection which comprises administering to the subject an amount of the pharmaceutical composition of claim 41 so as to alleviate the symptoms of the AIDS viral infection and thereby treating the subject.

55. A method for treating a subject suffering from a disorder associated with a TGF-ß which comprises administering to the subject an amount of mature TGF-ß3 so as to alleviate the symptoms of the disorder and thereby treating the subject.

56. The method of claim 55, wherein the disorder is a connective tissue disorder.

57. The method of claim 55, wherein the disorder is a neurological disorder.

58. The method of claim 55, wherein the neurological disorder is a demyelinating disease.

59. The method of claim 55, wherein the disorder is an immunosuppressive disorder.

60. The method of claim 55, wherein the disorder is an inflammatory disorder.

61. The method of claim 55, wherein the disorder is septic shock.

62. The method of claim 55, wherein the disorder is a bone disorder.

63. The method of claim 62, wherein the bone disorder is a bone fracture.

64. The method of claim 55, wherein the disorder is a dermatological disorder.

65. The method of claim 55, wherein the disorder is myocardial ischemia.

66. The method of claim 55, wherein the disorder is a myopathic disorder.

66. The method of claim 55, wherein the disorder is atherosclerosis.

67. The method of claim 55, wherein the disorder is an AIDS viral infection.

68. A method for treating a subject suffering from a disorder associated with a TGF-β which comprises administering to the subject an amount of TGF-β3 precursor so as to alleviate the symptoms of the disorder and thereby treating the subject.

69. The method of claim 68, wherein the disorder is a cancer.

70. The method of claim 68, wherein the disorder is a connective tissue disorder.

71. The method of claim 68, wherein the disorder is a neurological disorder.

72. The method of claim 70, wherein the neurological disorder is a demyelinating disease.

73. The method of claim 68, wherein the disorder is an immunosuppressive disorder.

74. The method of claim 68, wherein the disorder is an inflammatory disorder.

75. The method of claim 68, wherein the disorder is septic shock.

76. The method of claim 68, wherein the disorder is a bone disorder.

77. The method of claim 75, wherein the bone disorder is a bone fracture.

78. The method of claim 68, wherein the disorder is a dermatological disorder.

79. The method of claim 68, wherein the disorder is myocardial ischemia.

80. The method of claim 68, wherein the disorder is a myopathic disorder.

81. The method of claim 68, wherein the disorder is atherosclerosis.

82. The method of claim 68, wherein the disorder is an AIDS

viral infection.

83. A method for obtaining bone marrow substantially free of actively dividing tumor cells which comprises:

(a) contacting bone marrow containing normal hematopoietic cells and actively growing tumor cells with an effective amount of a TGF-ß such that the growth of the normal hematopoietic cells is temporarily inhibited;

(b) subsequently contacting bone marrow with a tumor inhibiting drug under conditions such that growth of tumors cells is permanently prevented; and

(d) culturing bone marrow so as to permit growth of normal hematopoietic cells thereby obtaining bone marrow substantially free of actively dividing tumor cells.

84. A method for obtaining bone marrow substantially free of actively dividing tumor cells which comprises:

(a) contacting bone marrow containing normal hematopoietic cells and actively growing tumor cells with an effective amount of a TGF-ß;

(b) culturing bone marrow of step (a) in the presence of TGF-ß for a suitable period under conditions such that terminal differentiation and clearance of tumor cells is permitted; and

(e) obtaining bone marrow substantially free of actively growing tumor cells.

85. The method of any of claims 83 or 84, wherein the TGF-ß is TGF-ß1.

86. The method of any of claims 83 or 84, wherein the TGF-ß is TGF-ß2.

87. The method of any of claims 83 or 84, wherein the TGF-ß is TGF-ß3.

88. A method for inhibiting cytotoxic poisoning of normal cells caused by chemotherapeutic agents which comprises contacting normal cells with an amount of a TGF-ß3 under conditions such that normal cell growth in the presence of chemotherapeutic agents is temporarily inhibited thereby inhibiting cytotoxic poisoning of normal cells.

FIGURE 1

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CCAGCAAAACCTGTTTAGACACATGGACAAGAATCCCAGCGCTACAAGGCACACAGTCCGCTTCTTCGTCCTCAGGGTTGCCAGCGCTTCCTGGAAGTCC
      10        20        30        40        50        60        70        80        90       100
TGAAGCTCTCGCAGTGCAGTGAGTTCATGCACCTTCTTGCCAAGCCTCAGTCTTTGGGATCTGGGGAGGCCGCCTGGTTTTCCTCCCTCCTTCTGCACGT
     110       120       130       140       150       160       170       180       190       200
                                                  Alu I                 M  K  M  H  L  Q  R  A  L  V  V  L  A
CTGCTGGGGTCTCTTCCTCTCCAGGCCTTGCCGTCCCCCTGGCCTCTCTTCCCAGCTCACACATGAAGATGCACTTGCAAAGGGCTCTGGTGGTCCTGGC
     210       220       230       240       250       260       270       280       290       300
  L  L  N  F  A  T  V  S  L  S  L  S  T  C  T  T  L  D  F  G  H  I  K  K  K  R  V  E  A  I  R  G  Q
CCTGCTGAACTTTGCCACGGTCAGCCTCTCTCTGTCCACTTGCACCACCTTGGACTTCGGCCACATCAAGAAGAAGAGGGTGGAAGCCATTAGGGGACAG
     310       320       330       340       350       360       370       380       390       400
  I  L  S  K  L  R  L  T  S  P  P  E  P  T  V  M  T  H  V  P  Y  Q  V  L  A  L  Y  N  S  T  R  E  L
ATCTTGAGCAAGCTCAGGCTCACCAGCCCCCCTGAGCCAACGGTGATGACCCACGTCCCCTATCAGGTCCTGGCCCTTTACAACAGCACCCGGGAGCTGC
     410       420       430       440       450       460       470       480       490       500
L  E  E  M  H  G  E  R  E  E  G  C  T  Q  E  N  T  E  S  E  Y  Y  A  K  E  I  H  K  F  D  M  I  Q  G
TGGAGGAGATGCATGGGGAGAGGGAGGAAGGCTGCACCCAGGAAAACACCGAGTCGGAATACTATGCCAAAGAAATCCATAAATTCGACATGATCCAGGG
     510       520       530       540       550       560       570       580       590       600
  L  A  E  H  N  E  L  A  V  C  P  K  G  I  T  S  K  V  F  R  F  N  V  S  S  V  E  K  N  R  T  N  L
GCTGGCGGAGCACAACGAACTGGCTGTCTGCCCTAAAGGAATTACCTCCAAGGTTTTCCGCTTCAATGTGTCCTCAGTGGAGAAAAATAGAACCAACCTA
     610       620       630       640       650       660       670       680       690       700
  F  R  A  E  F  R  V  L  R  V  P  N  P  S  S  K  R  N  E  Q  R  I  E  L  F  Q  I  L  R  P  D  E  H
TTCCGAGCAGAATTCCGGGTCTTGCGGGTGCCCAACCCCAGCTCTAAGCGGAATGAGCAGAGGATCGAGCTCTTCCAGATCCTTCGGCCAGATGAGCACA
     710       720       730       740       750       760       770       780       790       800
I  A  K  Q  R  Y  I  G  G  K  N  L  P  T  R  G  T  A  E  W  L  S  F  D  V  T  D  T  V  R  E  W  L  L
TTGCCAAACAGCGCTATATCGGTGGCAAGAATCTGCCCACACGGGGCACTGCCGAGTGGCTGTCCTTTGATGTCACTGACACTGTGCGTGAGTGGCTGTT
     810       820       830       840       850       860       870       880       890       900
  R  R  E  S  N  L  G  L  E  I  S  I  H  C  P  C  H  T  F  Q  P  N  G  D  I  L  E  N  I  H  E  V  M
GAGAAGAGAGTCCAACTTAGGTCTAGAAATCAGCATTCACTGTCCATGTCACACCTTTCAGCCCAATGGAGATATCCTGGAAAACATTCACGAGGTGATG
     910       920       930       940       950       960       970       980       990      1000
  E  I  K  F  K  G  V  D  N  E  D  D  H  G  R  G  D  L  G  R  L  K  K  Q  K  D  H  H  N  P  H  L  I
GAAATCAAATTCAAAGGCGTGGACAATGAGGATGACCATGGCCGTGGAGATCTGGGGCGCCTCAAGAAGCAGAAGGATCACCACAACCCTCATCTAATCC
     1010      1020      1030      1040      1050      1060  *   1070      1080      1090      1100
L  M  M  I  P  P  H  R  L  D  N  P  G  Q  G  G  Q  R  K  K  R  A  L  D  T  N  Y  C  F  R  N  L  E  E
TCATGATGATTCCCCCACACCGGCTCGACAACCCGGGCCAGGGGGGTCAGAGGAAGAAGCGGGCTTTGGACACCAATTACTGCTTCCGCAACTTGGAGGA
     1110      1120      1130      1140      1150      1160      1170      1180      1190      1200
  N  C  C  V  R  P  L  Y  I  D  F  R  Q  D  L  G  W  K  W  V  H  E  P  K  G  Y  Y  A  N  F  C  S  G
GAACTGCTGTGTGCGCCCCCTCTACATTGACTTCCGACAGGATCTGGGCTGGAAGTGGGTCCATGAACCTAAGGGCTACTATGCCAACTTCTGCTCAGGC
     1210      1220      1230      1240      1250      1260      1270      1280      1290      1300
```

FIGURE 1 (CONTINUATION)

```
 P  C  P  Y  L  R  S  A  D  T  T  H  S  T  V  L  G  L  Y  N  T  L  N  P  E  A  S  A  S  P  C  C  V
CCTTGCCCATACCTCCGCAGTGCAGACACAACCCACAGCACGGTGCTGGGACTGTACAACACTCTGAACCCTGAAGCATCTGCCTCGCCTTGCTGCGTGC
      1310       1320       1330       1340       1350       1360       1370       1380       1390       1400
 P  Q  D  L  E  P  L  T  I  L  Y  Y  V  G  R  T  P  K  V  E  Q  L  S  N  M  V  V  K  S  C  K  C  S
CCCAGGACCTGGAGCCCCTGACCATCCTGTACTATGTTGGGAGGACCCCCAAAGTGGAGCAGCTCTCCAACATGGTGGTGAAGTCTTGTAAATGTAGCTG
      1410       1420       1430       1440       1450       1460       1470       1480       1490       1500
AGACCCCACGTGCGACAGAGAGAGGGGAGAGAGAACCACCACTGCCTGACTGCCCGCTCCTCGGGAAACACACAAGCAACAAACCTCACTGAGAGGCCTG
      1510       1520       1530       1540       1550       1560       1570       1580       1590       1600
GAGCCCACAACCTTCGGCTCCGGGCAAATGGCTGAGATGGAGGTTTCCTTTTGGAACATTTCTTTCTTGCTGGCTCTGAGAATCACGGTGGTAAAGAAAG
      1610       1620       1630       1640       1650       Hga I  1670       1680       1690       1700
TGTGGGTTTGGTTAGAGGAAGGCTGAACTCTTCAGAACACACAGACTTTCTGTGACGCAGACAGAGGGGATGGGGATAGAGGAAAGGGATGGTAAGTTGA
      1710       1720       1730       1740       1750       1760       1770       1780       1790       1800
GATGTTGTGTGGCAATGGGATTTGGGCTACCCTAAAGGGAGAAGGAAGGGCAGAGAATGGCTGGGTCAGGGCCAGACTGGAAGACACTTCAGATCTGAGG
      1810       1820       1830       1840       1850       1860       1870       1880       1890       1900
TTGGATTTGCTCATTGCTGTACCACATCTGCTCTAGGGAATCTGGATTATGTTATACAAGGCAAGCATTTTTTTTTTTTTTTAAAGACAGGTTACGAAGA
      1910       1920       1930       1940       1950       1960       1970       1980       1990       2000
CAAAGTCCCAGAATTGTATCTCATACTGTCTGGGATTAAGGGCAAATCTATTACTTTTGCAAACTGTCCTCTACATCAATTAACATCGTGGGTCACTACA
      2010       2020       2030       2040       2050       2060       2070       2080       2090       2100
GGGAGAAAATCCAGGTCATGCAGTTCCTGGCCCATCAACTGTATTGGGCCTTTTGGATATGCTGAACGCAGAAGAAAGGGTGGAAATCAACCCTCTCCTG
      2110       2120       2130       2140       2150       2160       2170       2180       2190       2200
TCTGCCTCTGGGTCCCTCCTCTCACCTCTCCCTCGATCATATTTCCCCTTGGACACTTGGTTAGACGCCTTCCAGGTCAGGATGCACATTTCTGGATTGT
      2210       2220       2230       2240       2250       2260       2270       2280       2290       2300
GGTTCCATGCAGGGTTGGGGCATTATGGGTTCTTCCCCCACTTCCCCTCCAAGACCCTGTGTTCATTTGGTGTTCCTGGAAGCAGGTGCGACAACATGTG
      2310       2320       2330       2340       2350       2360       2370       2380       2390       2400
AGGCATTCGGGGAAGCTCGACATGTGCCACACAGTGACTTGGCCCCAGACGCATAGACTGAGGTATAAAGACAAGTATGAATATTACTCTCAAAATCTTT
      2410       2420       2430       2440       2450       2460       2470       2480       2490       2500
GTATAAATAAATATTTTTGGGGCATCCTG poly(A)
      2510       2520
```

FIGURE 2

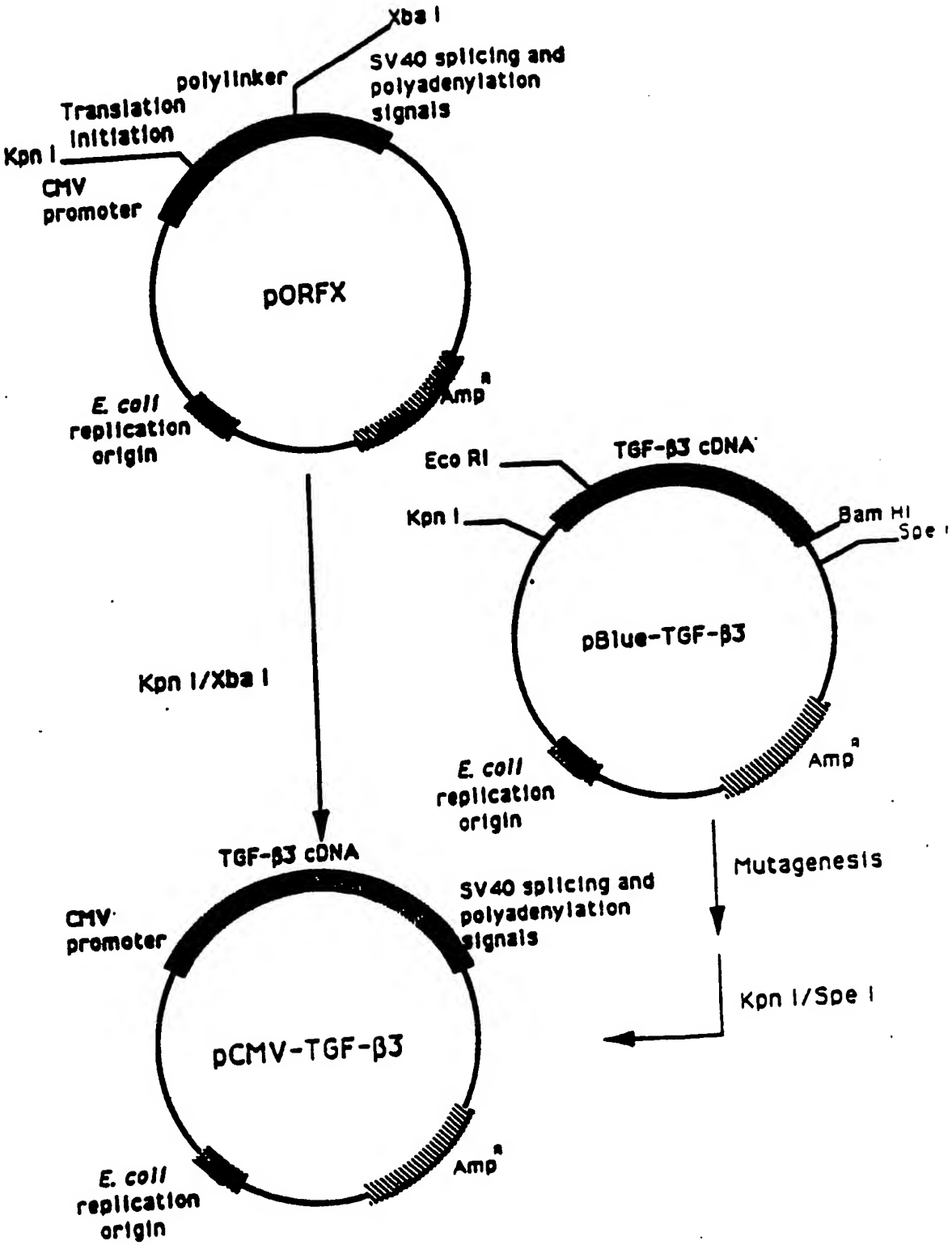
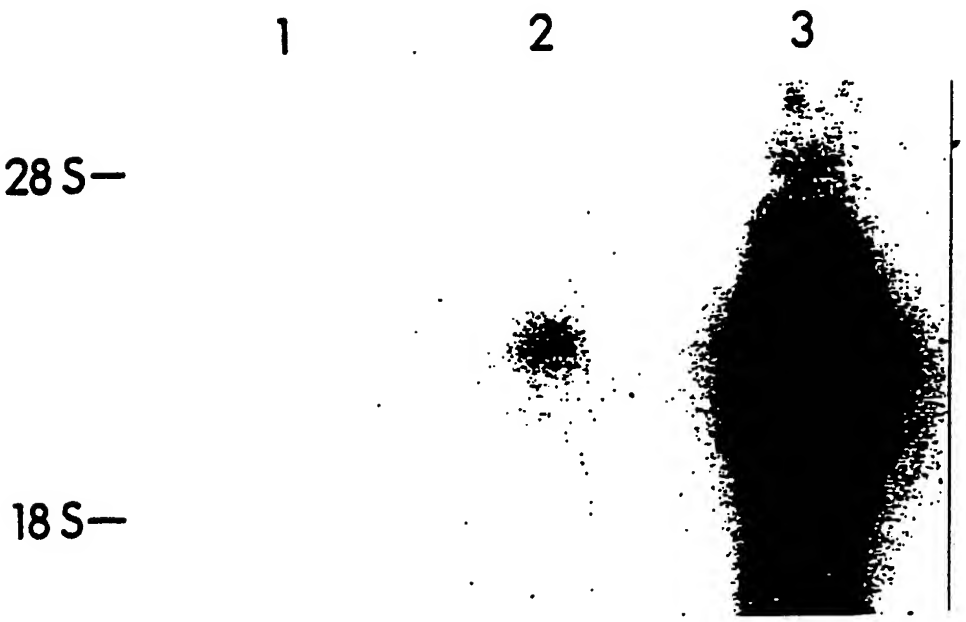

Xba I
SV40 splicing and polyadenylation signals
polylinker
Translation initiation
Kpn I
CMV promoter
pORFX
E. coli replication origin
Amp^R
Eco RI
TGF-β3 cDNA
Kpn I
Bam HI
Spe I
pBlue-TGF-β3
E. coli replication origin
Amp^R
Kpn I/Xba I
Mutagenesis
Kpn I/Spe I
TGF-β3 cDNA
SV40 splicing and polyadenylation signals
CMV promoter
pCMV-TGF-β3
E. coli replication origin
Amp^R

FIGURE 3



5/23

FIGURE 4

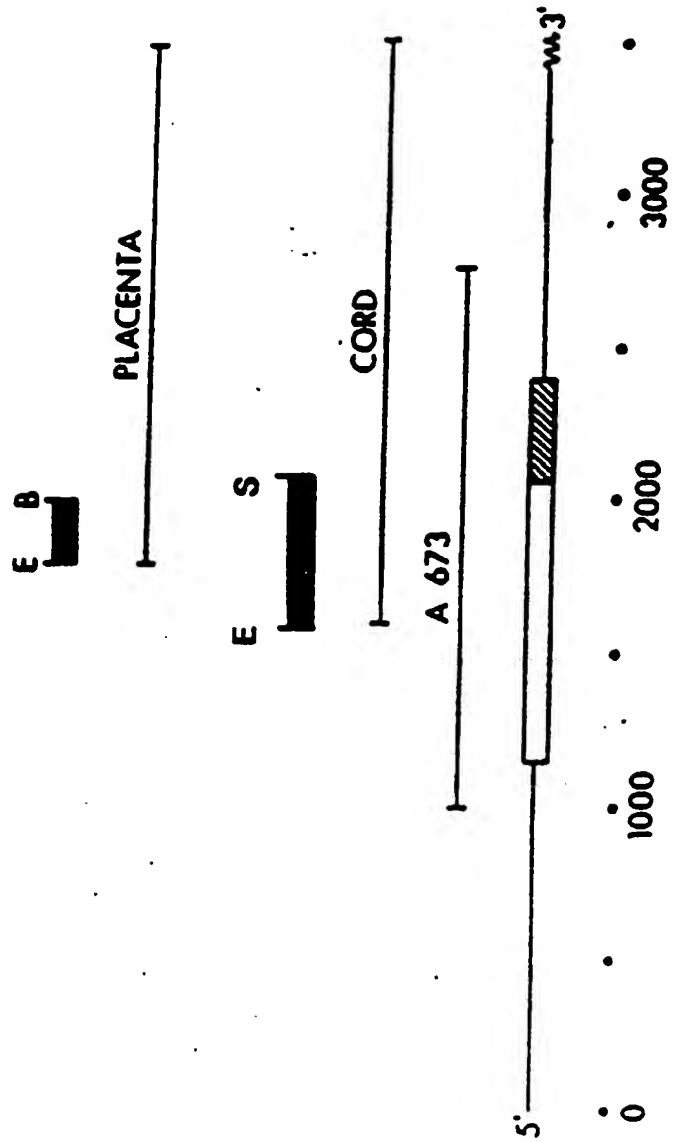


FIGURE 5A

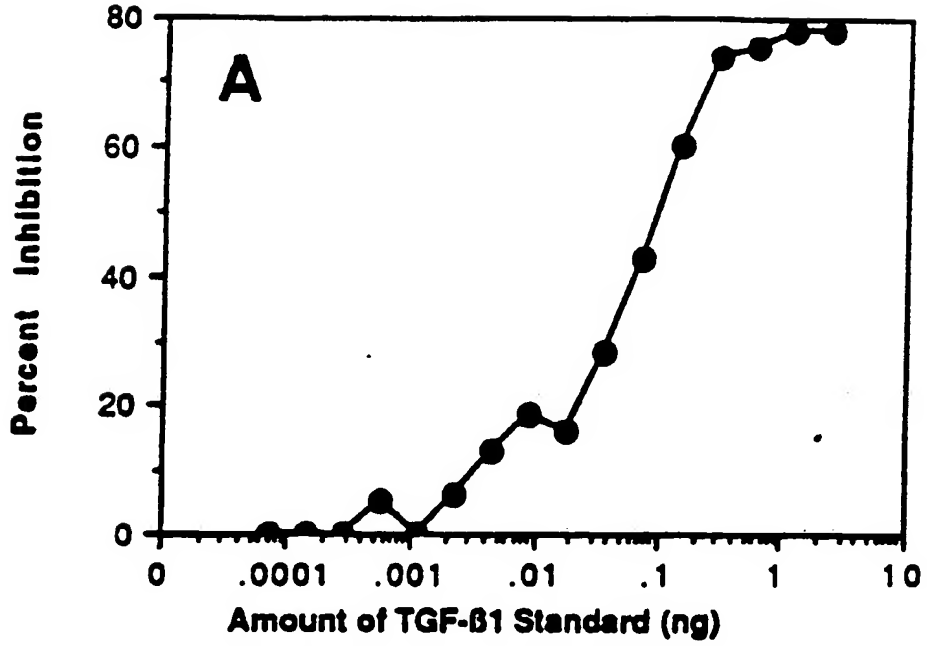

A
Percent Inhibition
80
60
40
20
0
0
.0001
.001
.01
.1
1
10
Amount of TGF-ß1 Standard (ng)

FIGURE 5B

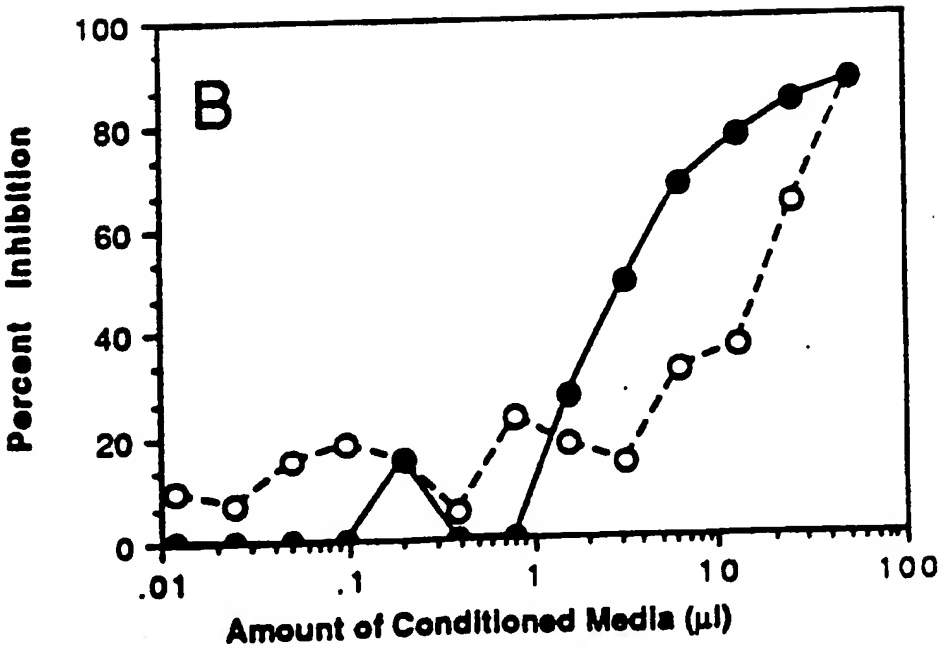

B
Percent Inhibition
100
80
60
40
20
0
.01
.1
1
10
100
Amount of Conditioned Media (µl)

FIGURE 6

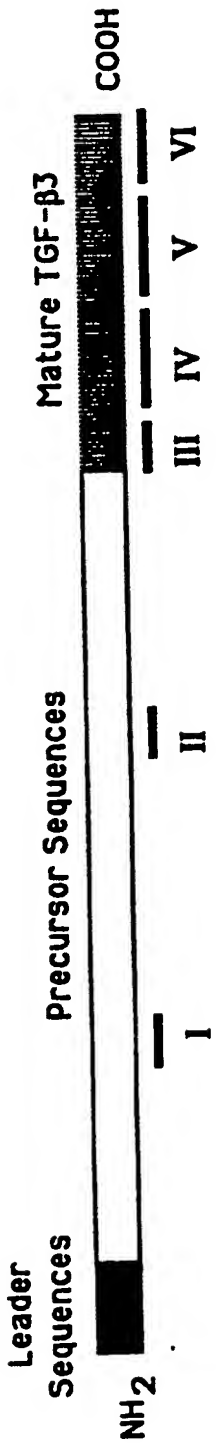
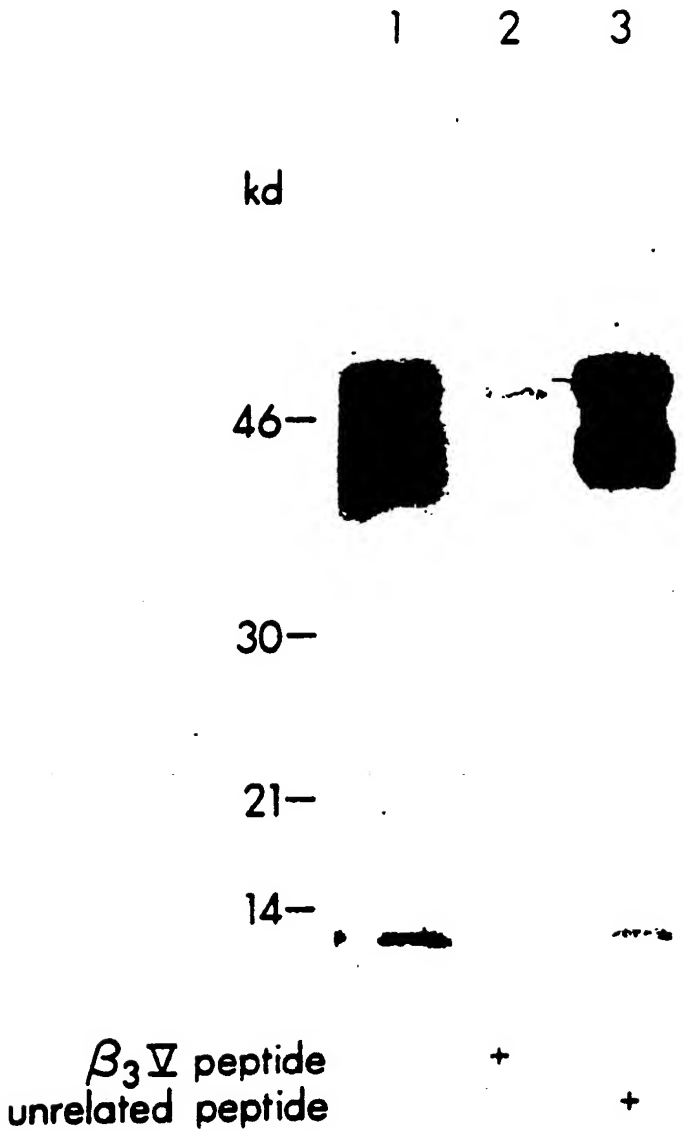
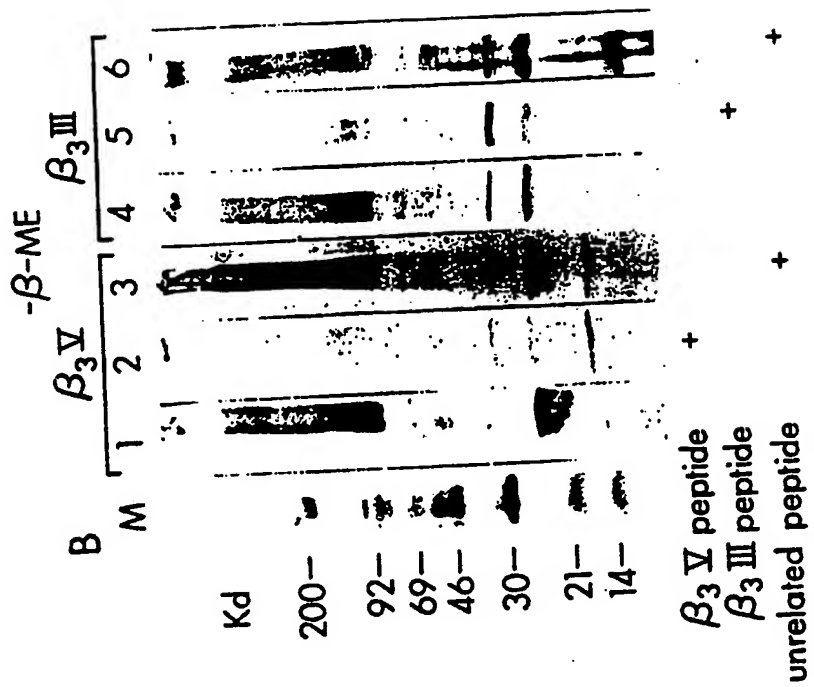

COOH
Mature TGF-β3
VI
V
IV
III
II
I
Precursor Sequences
Leader Sequences
NH2

FIGURE 7




B
M
-β-ME
β3V
β3III
1
2
3
4
5
6
Kd
200—
92—
69—
46—
30—
21—
14—
β3 V peptide
β3 III peptide
unrelated peptide

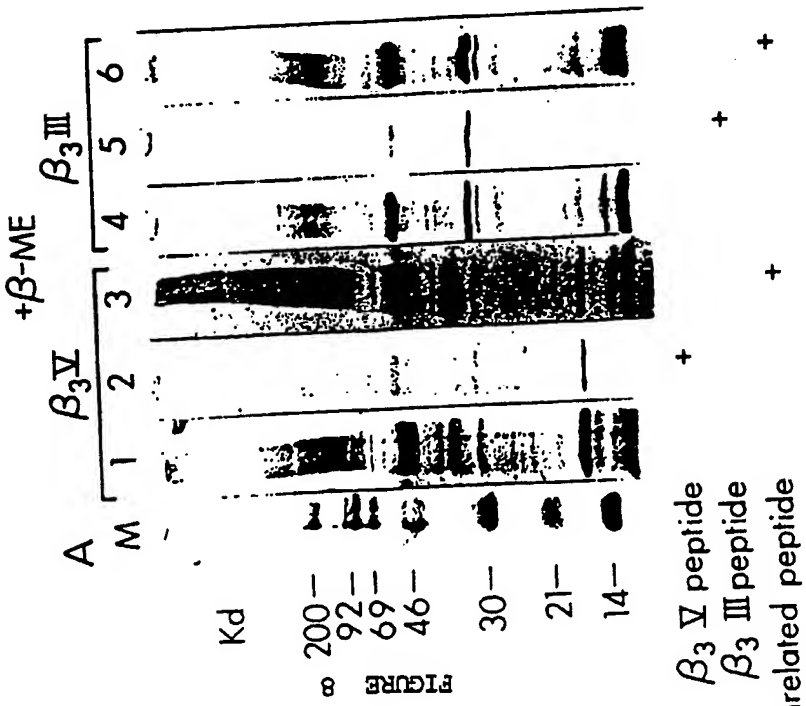

A
M
+β-ME
β3V
β3III
1
2
3
4
5
6
Kd
200—
92—
69—
46—
30—
21—
14—
β3 V peptide
β3 III peptide
unrelated peptide

FIGURE 8

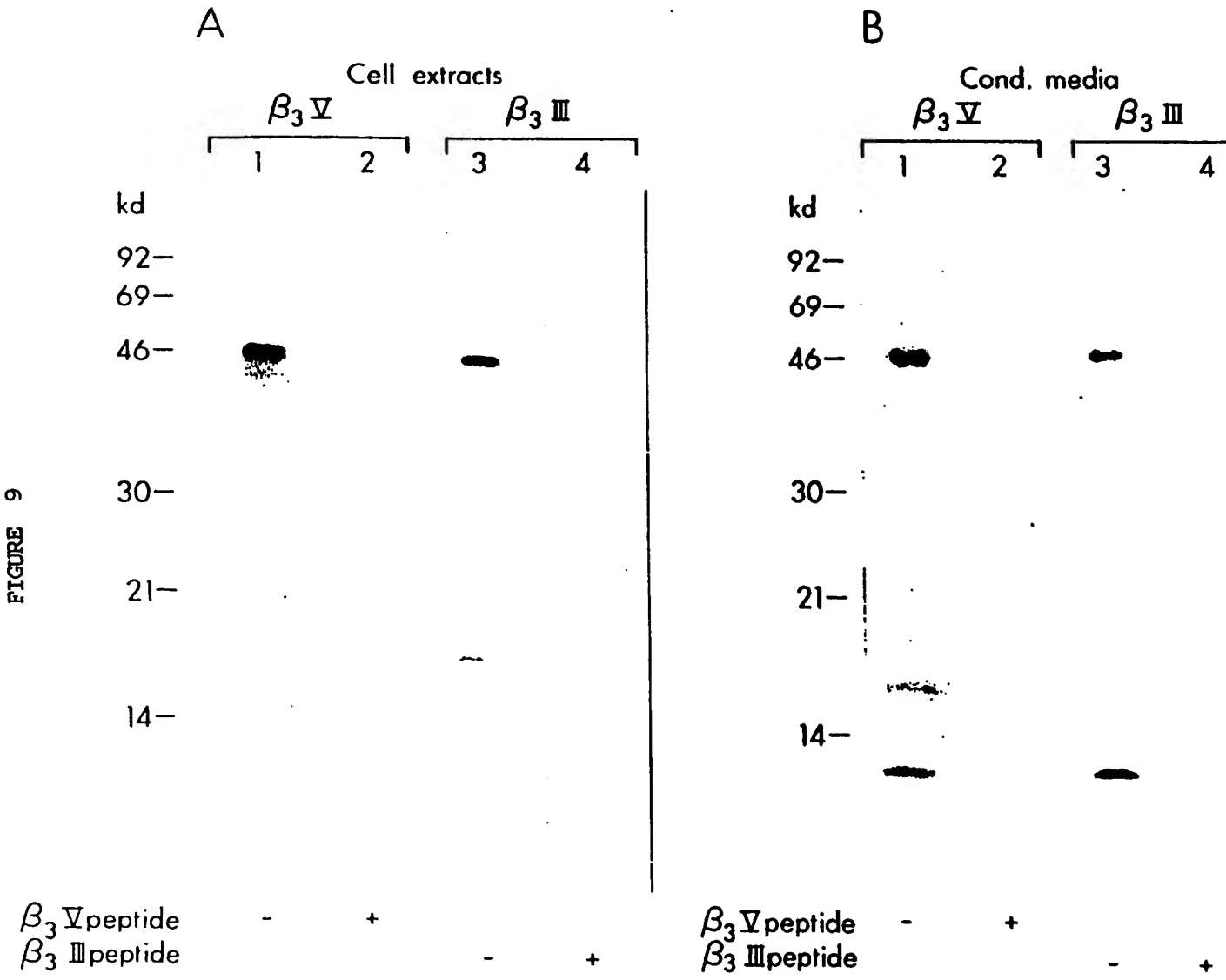

A
Cell extracts
β_3 V
β_3 III
1
2
3
4
kd
92—
69—
46—
30—
21—
14—
β_3 Vpeptide - +
β_3 IIIpeptide - +
B
Cond. media
β_3 V
β_3 III
1
2
3
4
kd
92—
69—
46—
30—
21—
14—
β_3 Vpeptide - +
β_3 IIIpeptide - +

FIGURE 9

FIGURE 10A

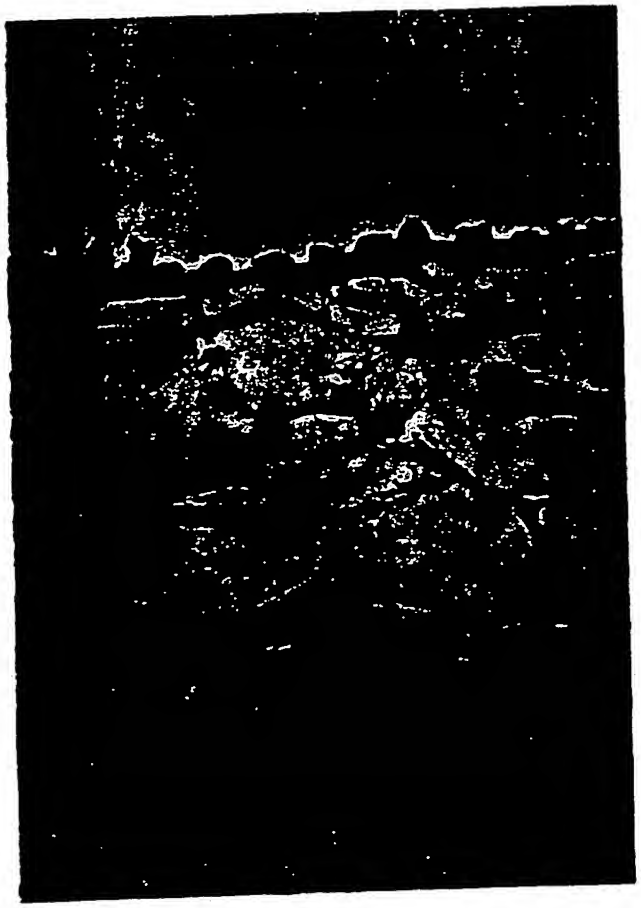


FIGURE 10B

EXHIBIT 10C

EXHIBIT 10D

FIGURE 11A

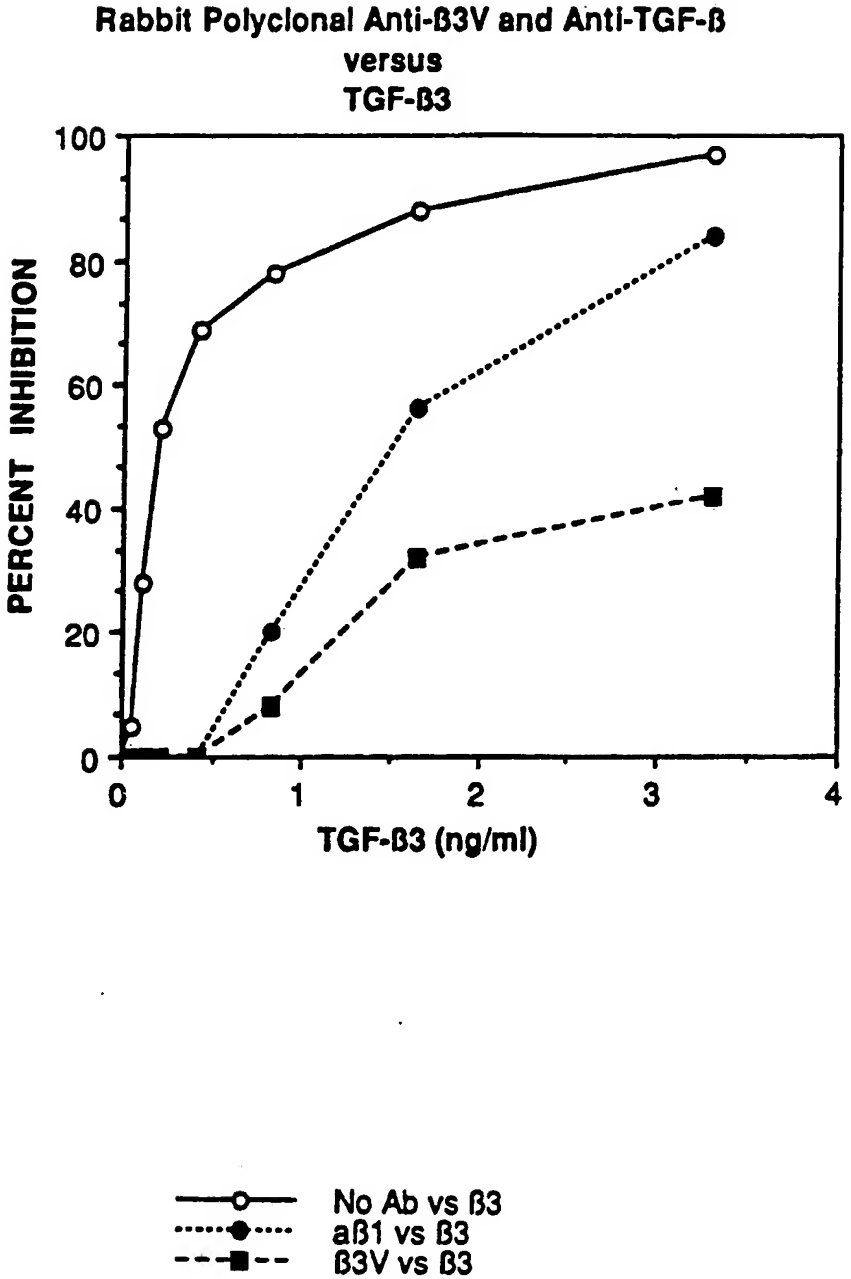

Rabbit Polyclonal Anti-ß3V and Anti-TGF-ß
versus
TGF-ß3
PERCENT INHIBITION
100
80
60
40
20
0
0
1
2
3
4
TGF-ß3 (ng/ml)
No Ab vs ß3
aß1 vs ß3
ß3V vs ß3

FIGURE 11B

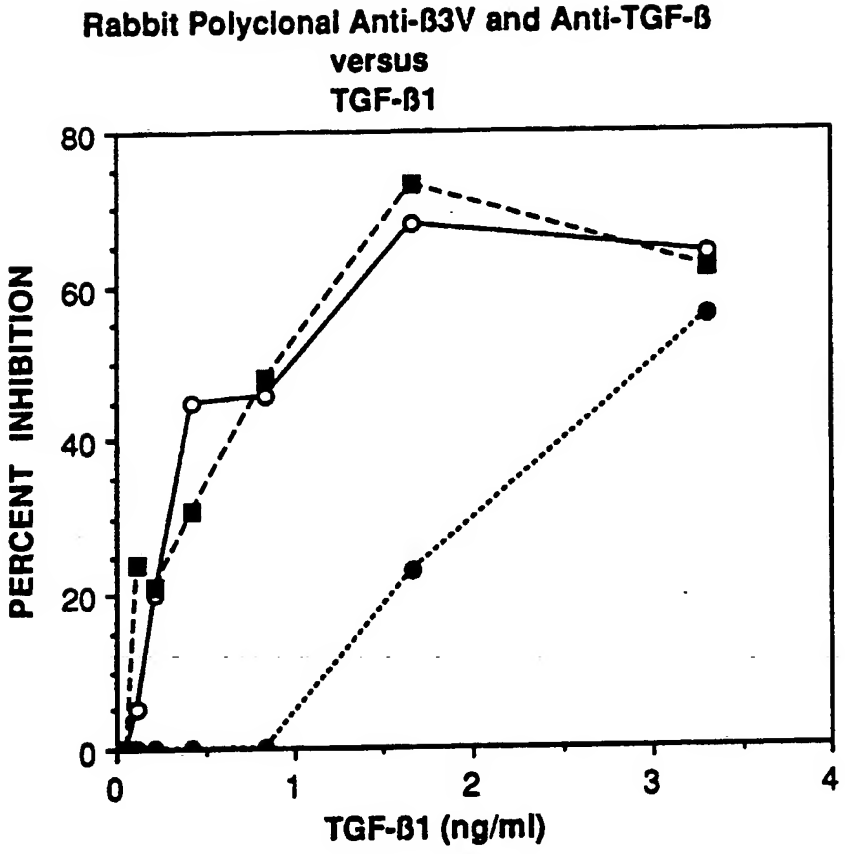

Rabbit Polyclonal Anti-ß3V and Anti-TGF-ß
versus
TGF-ß1
PERCENT INHIBITION
80
60
40
20
0
0
1
2
3
4
TGF-ß1 (ng/ml)
No Ab vs ß1
aß1 vs ß1
ß3V vs ß1

FIGURE 11C

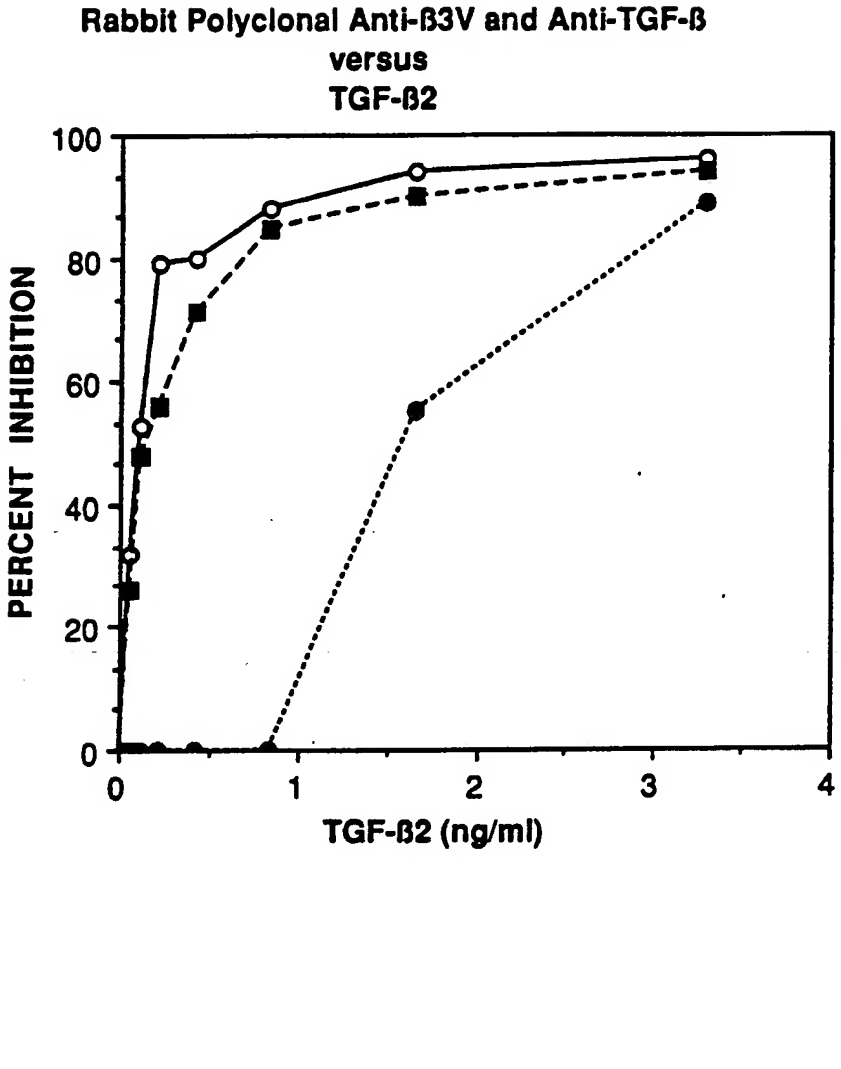

Rabbit Polyclonal Anti-ß3V and Anti-TGF-ß
versus
TGF-ß2
PERCENT INHIBITION
100
80
60
40
20
0
0
1
2
3
4
TGF-ß2 (ng/ml)
No Ab vs ß2
aß1 vs ß2
ß3V vs ß2

FIGURE 12A

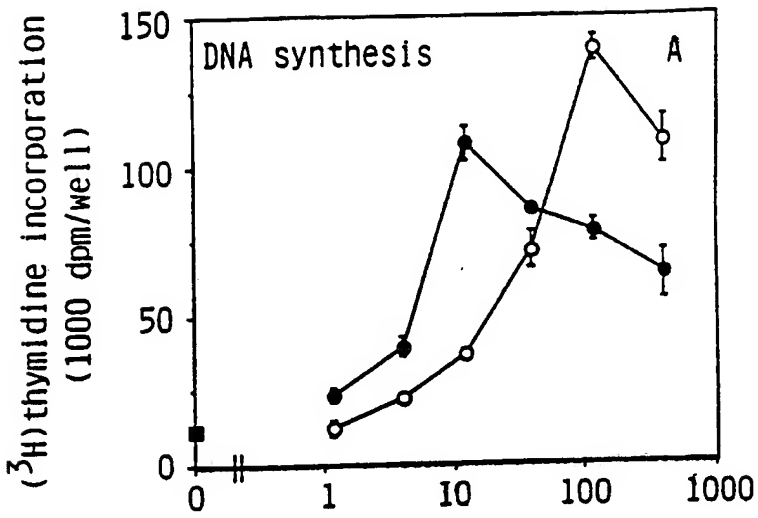

DNA synthesis
A
(^{3}H)thymidine incorporation
(1000 dpm/well)
150
100
50
0
0
1
10
100
1000

FIGURE 12B

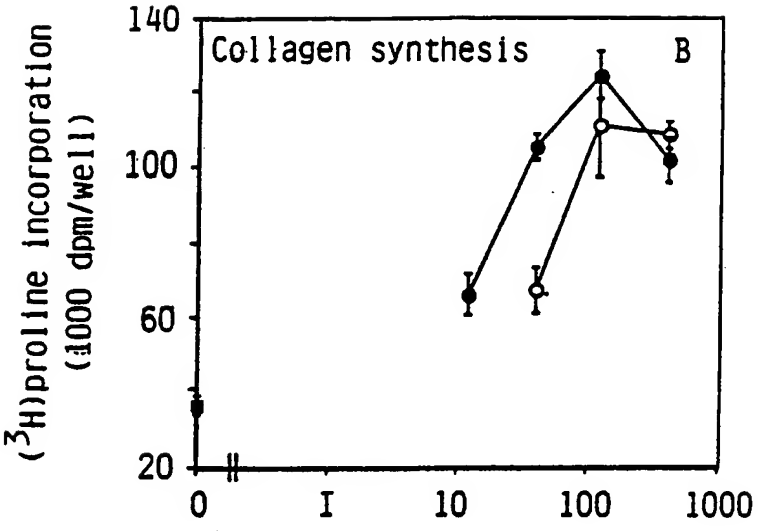
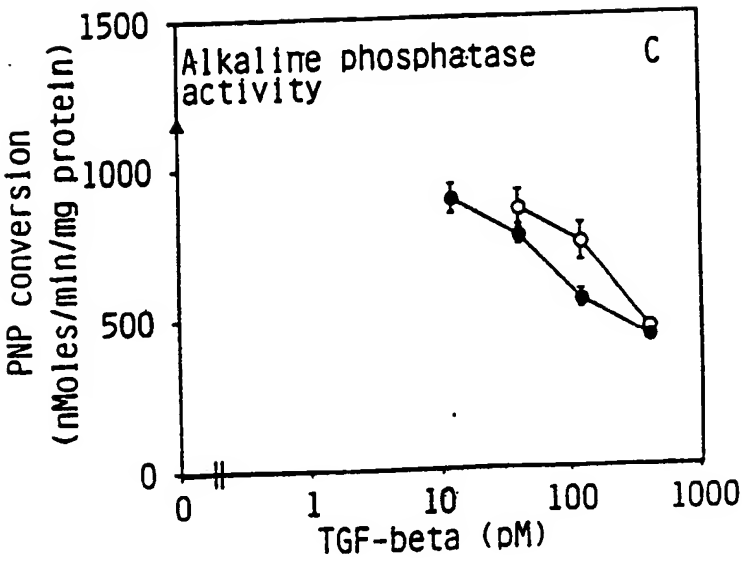

Collagen synthesis
B
(^{3}H)proline incorporation
(1000 dpm/well)
140
100
60
20
0
I
10
100
1000

FIGURE 12C


Alkaline phosphatase activity
C
PNP conversion (nMoles/min/mg protein)
1500
1000
500
0
0
1
10
100
1000
TGF-beta (pM)

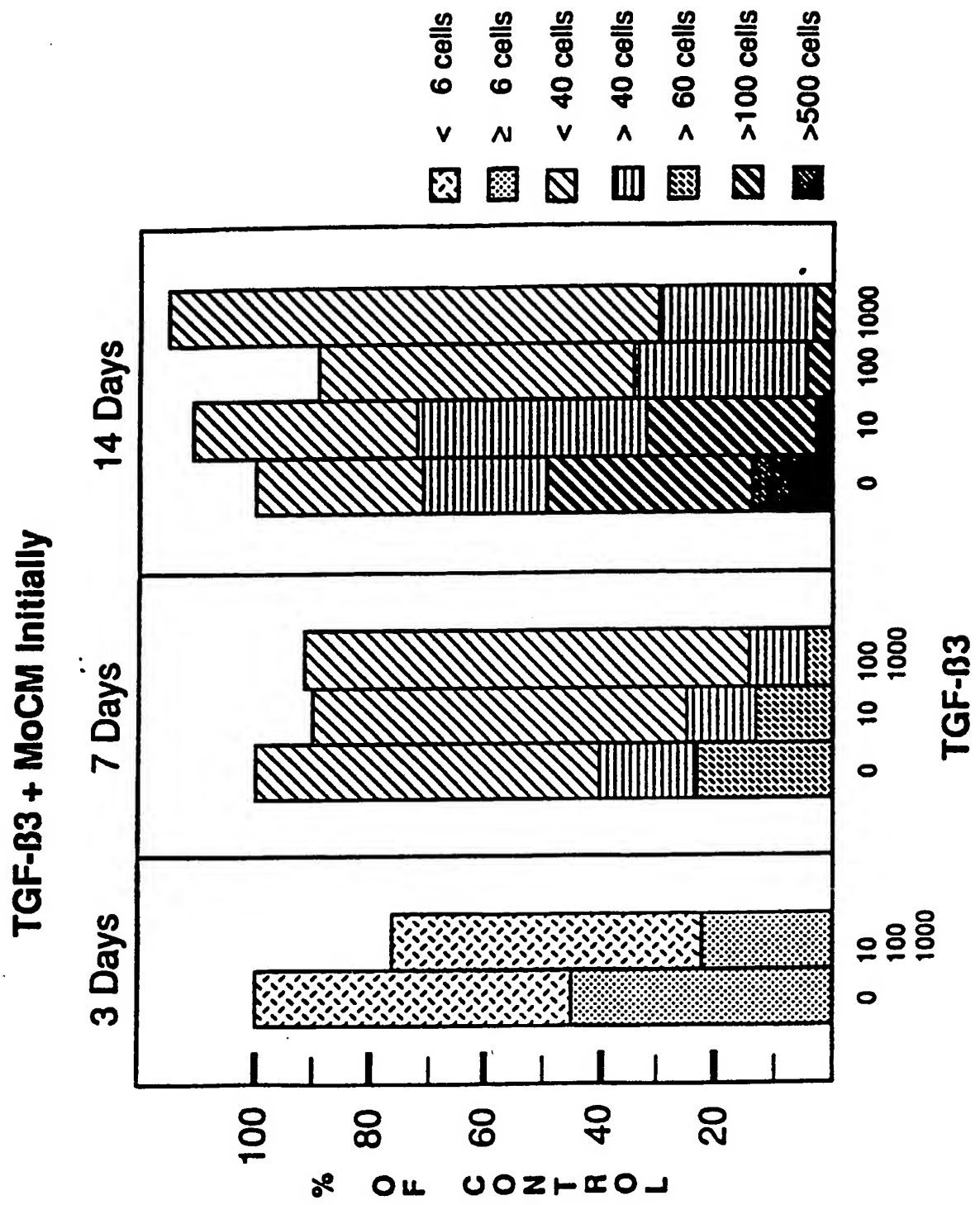
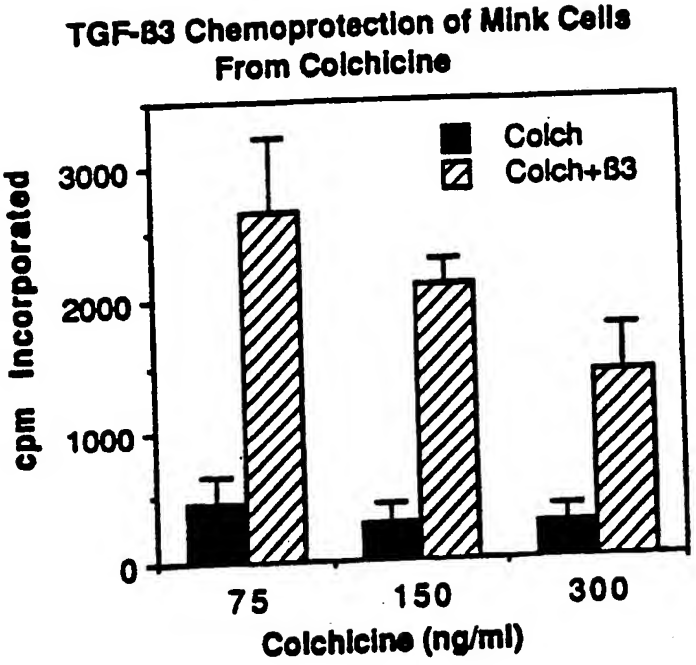
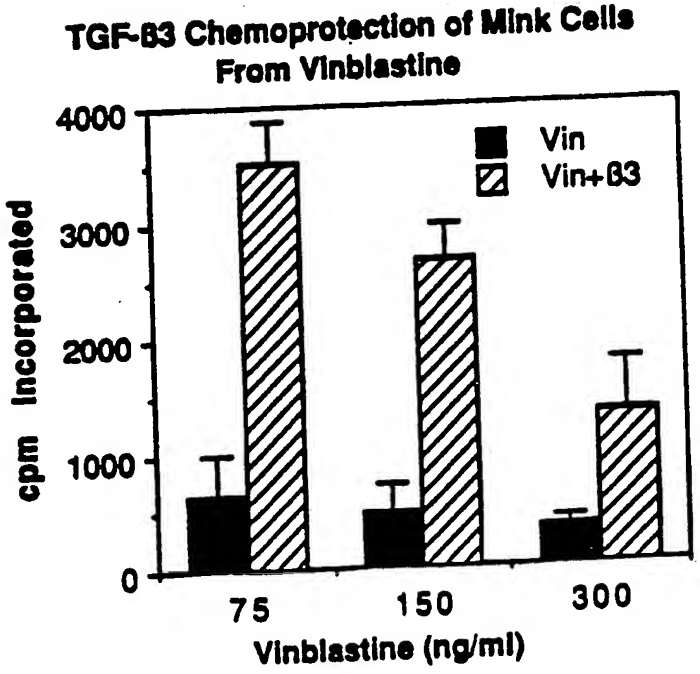

< 6 cells
≥ 6 cells
< 40 cells
> 40 cells
> 60 cells
>100 cells
>500 cells
TGF-β3 + MoCM Initially
3 Days
7 Days
14 Days
0 10 100 1000
TGF-β3
% OF CONTROL
100
80
60
40
20

FIGURE 14


TGF-β3 Chemoprotection of Mink Cells From Colchicine
Colch
Colch+β3
cpm Incorporated
3000
2000
1000
0
75
150
300
Colchicine (ng/ml)


TGF-β3 Chemoprotection of Mink Cells From Vinblastine
Vin
Vin+β3
cpm Incorporated
4000
3000
2000
1000
0
75
150
300
Vinblastine (ng/ml)

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US91/04540

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): C07K 15/28; A61K 37/36; C12Q 1/25
US.CL.: 424/85.8; 514/8; 530/387; 435/7.92

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
U.S.	530/387,388; 424/85.8,85.91; 514/2,8,12 435/7.92,240.2,240.21; 436/542,548

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

Databses: Dialog (Files 5,155, 172, 351); LISPTO Automated Patent System (File USPAT, 1971-1991).

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
Y	EMBOJOURNAL, Vol. 7, No. 12. issued 1988, Dernyck et al, "A newtype of transforming growth factor-B, TGF-B3," pages 3737-3743, see entire document.	1-89
Y	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES USA, Vol. 85, issued July 1988, Ten Dijke et al., "Identification of another member of the transforming growth factor type B gene family," pages 4715-4719, see entire document.	1-89
Y,P	MOLECULAR AND CELLULAR BIOLOGY, Vol. 10 No 9, issued September 1990, Ten Dijke et al., "Recombinant Transforming Growth Factor Type B3: Biological Activities and Receptor-Binding Properties in Isolated Bone Cells," page 4473-4479, see entire document.	1-89

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search

06 September 1991

Date of Mailing of this International Search Report

23 OCT 1991

International Searching Authority

ISA/US

Signature of Authorized Officer

Robert D. Budens tf

Form PCT/ISA/210 (second sheet) (Rev.11-87)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y,P	JOURNAL OF BIOLOGICAL CHEMISTRY, Vol. 265, No. 33 issued 25 November 1990, Cheifetz et al.," Distinct Transforming Growth Factor-B(TGF-B) Receptor Subsets as Determinants of cellular Responsiveness to Three TGF-B Isoforms," pages 20533-20538. see entire document.	1-89
Y	CELL Vol. 49, issued 22 May 1987, Massague et al.. "The TGF-B Family of Growth and Differentiation Factors," pages 437-438. see entire document.	1-89
Y	CANCER RESEARCH, Vol. 45, issued June 1985, Iwata et al.. "Isolation of Tumor Cell Growt Inhibiting Factors from a Human Rhabdomyosarcoma Cell Line," pages 2689-2694, see entire document.	41-45, 55,69-70.
Y	CELL, Vol. 52, issued 12 February 1988, Rossi et al., "A Nuclear Factor 1 Binding Site Mediates the Transcriptional Activation of A Type 7 Collagen Promotor by Transforming Growth Factor-B" pages 405-414. See entire document.	13,16,18-20,25,30 33,40-44, 46.49.53 56.60,62 63.68.71 75,77,78 79,
Y	JOURNAL OF BIOLOGICAL CHEMISTRY, Vol. 261, No. 35, issued 15 December 1986, Florini et al., "Transforming Growth Factor-B: A very potent inhibitor of Myoblost Differentiation, Indentical to the Differentiation Inhibitor Secreted by Buffalo Rat Liver Cells." pages 16509-16513, see entire document.	14-16,28 30,41-44, 46,50-51, 56,65-66, 70,80-81.
Y	JOURNAL OF CELL BIOLOGY, Vol. 107, issued issued August 1988. Owens et al., "Transforming Growth Factor-B-Induced Growth Inhibition and Cellular Hypertrophy in Cultured Vascular Smooth Muscle Cells," pages 771-780, see entire document.	14-16.28-30,41-44, 46,50-51 56,65-66, 70, 80-81

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	JOURNAL OF CELL BIOLOGY, Vol. 110, issued April 1990, Davis et al., "Platelet-derived Growth Factors and Fibioblast Growth Factors Are Mitogens for Rat Schwann Cells," pages 1353-1360, see entire document.	17,32,41-44 47,57-58,72 73
Y	JOURNAL OF CELL BIOLOGY, Vol. 105, issued September 1987, Sporn et al., "Some Recent Advances in the Chemistry and Biology of Transforming Growth Factor-Beta," pages 1039-1045. see entire document.	1-89
Y	NATURE Vol. 299, issued 14 October 1982, Lerner. "Tapping the Immunological Repertoire to Produce Antibodies of Redetermined Specificity," pages 592-596, see entire document.	1-40
Y	ADVANCES IN IMMUNOLOGY, Vol. 36, issued 1984, Lerner, "Antibodies of Predetermined Specificity in Biology and Medicine;" pages 1-44, see entire document.	1-40

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers . because they relate to subject matter [12] not required to be searched by this Authority, namely:

2. ☐ Claim numbers , because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful International search can be carried out [13], specifically:

3. ☐ Claim numbers_____, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

See attached sheets

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application. Telephone practice
2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

In the examination of international applications filed under the Patent Cooperation Treaty, PCT Rule 13.1 states that the international application shall relate to one invention only or to a group of inventions so linked as to form "a single general inventive concept."

PCT Rule 13.2 indicates that this shall be construed as permitting, in particular, one of the following three possible combinations of the claimed invention:

(1) a product, a process specifically adapted for the manufacture of said product and a use of said product, or

(2) a process, and an apparatus or means specifically designed for carrying out said process, or

(3) a product, a process specially adapted for the manufacture of said product and an apparatus or means designed for carrying out the process.

Additionally, current United States Patent and Trademark Office restriction practice permits the following combinations of the claimed invention:

(4) a product, and a process specifically adapted for the manufacture of said product, and

(5) a product, and a use of the said product, as where said use as claimed cannot be practiced with another materially different product.

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claims 1-22, a first product and method of use, drawn to antibodies to TGF-β3 and methods of use.

Group II, claims 23-40, a second method, drawn to methods of treatment using TGF-β3.

Group III, claims 41-44, a second product, drawn to pharmaceutical compositions.

Group IV, claims 45-54, a third method, drawn to methods of treatment using pro region of TGF-β3.

Group V, claims 55-68, a fourth method, drawn to methods of treatment using mature TGF-β3.

Group VI, claims 69-83, a fifth method, drawn to methods of treatment using TGF-β3 precursor.

Group VII, claims 84-88, a sixth method, drawn to methods of obtaining bone marrow.

Group VIII, claim 89, a seventh method, drawn to a method of inhibiting cytotoxicity.

Group IX, claims 11, 26, 48, 59 and 74, a second specie of disorder, drawn to an immune suppressive disease.

Group X, claims 12, 27, 54, 68 and 83, a third specie of disorder, drawn to an AIDS viral infection.

Group XI, claims 13, 64 and 79, a fourth specie of disorder, drawn to a dermatological disorder.

Group XII, claims 14, 28, 50, 65 and 80, a fifth specie of disorder, drawn to myocardial ischemia.

Group XIII, claims 15, 29, 51, 66 and 81, a sixth specie of disorder, drawn to a myopathic disorder.

Group XIV, claims 16, 30, 46, 56 and 71, a seventh specie of disorder, drawn to a connective tissue disorder.

Group XV, claims 17, 32, 47 and 72, an eighth specie of disorder, drawn to a neurological disorder.

Group XVI, claims 19, 43 and 87, a second specie of TGF-β, drawn to TGF-β2.

Group XVII, claims 20, 44 and 88, a third specie of TGF-β, drawn to TGF-β3.

The inventions listed as Groups I-XVII do not meet the requirements for Unity of Invention for the following reasons:

The products of Groups I and III differ one from another in their physical properties such as chemical structure, primary sequence and molecular weight and are not so linked as to form a single general inventive concept.

The methods of Groups I and (II and IV-VIII) each differ one from another in method steps, reagents, and utility and are not so linked as to form a single general inventive concept.

The species of Groups IX-XV differ one from another in their pathologic mechanisms and etiology and are not so

linked as to form a single inventive concept.

The specie of Groups XVI-XVII differ one from another in chemical structure and physical properties such as primary sequence and molecular weight and are not so linked as to form a single general inventive concept.

During a telephonic requirement for election, on August 20, 1991, applicant's representative, Sarah Adriano, elected the invention of all the Groups for examination.